

ASSESSING THE EFFECTS OF FLUOXETINE ON THE GUT MICROBIOME OF  
FATHEAD MINNOW (PIMEPHALES PROMELAS)

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By

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## ABSTRACT

The understanding and importance of incorporating the microbiome as a measurable indicator of toxicity has only recently come about in the scientific discipline of ecotoxicology. The microbiome, specifically the gut microbiome, plays a critical role in host homeostasis where it can communicate, interact and influence the nervous, immune and gastrointestinal systems as well as the brain. The microbiome holds importance in ecotoxicology studies as microbiota can detoxify and activate toxicants and, if perturbed by contaminants, influence the host response to these compounds. Given some gut flora can influence neurochemicals, synthesize biogenic amines and express neurotransmitter:sodium symporters, it is valuable to understand how antidepressants, specifically selective serotonin reuptake inhibitors (SSRIs), can affect the gut microbiome. SSRIs are known contaminants found in aquatic environments, which has led to an abundance of ecotoxicology studies covering behavior, reproduction, and developmental research on aquatic organisms. However, no study has yet been identified assessing the gut microbial response to SSRIs in the field of ecotoxicology such as within fish. A preliminary study was first conducted to identify if fluoxetine, a common SSRI, can impede growth of bacterial cells cultured from the gut of fathead minnow (*Pimephales promelas*). This was an *in vitro* study, where bacterial cells were inoculated with a serial dilution ranging from 0.015 to 128 µg/mL of fluoxetine. At nominal fluoxetine concentrations of 32, 64 and 128 µg/mL, growth of bacterial cells was found to indeed be inhibited. With preliminary results identifying inhibition of growth due to fluoxetine exposure, an *in vivo* study followed where fathead minnows were exposed to three concentrations of fluoxetine or a control. Nominal concentrations consisted of 0.01, 10 and 100 µg/L. After a 28-day exposure to aqueous fluoxetine, fish were euthanized, intestines were extracted and DNA extraction, amplification, purification and sequencing was conducted on the 16S rRNA gene to identify the abundance and diversity of the gut microbiome. Results indicated that the highest exposed group held a significantly altered gut microbiome compared to the control, low and middle groups. It was also found that male and female fish hold distinct gut microbiomes. These findings are the first to identify that SSRIs can influence the gut microbiome of fish, although at a concentration much higher than known environmental concentrations. While the results are inconclusive at lower concentrations more commonly measured in the aquatic environment, insight still may be gained into how potential mixtures and continuous persistence of SSRIs may impact the gut microbiome of fish long-term. This research

can also help interpret how such concentrations of fluoxetine may influence the gut microbiome of other organisms using the cross-species extrapolation hypothesis to predict similar effects on evolutionarily conserved molecular targets. Possible next steps would be to perform a mixed-omics approach to accurately understand not only “who is there” but “what they are doing”; essentially, how the gut microbiota is functioning and interacting with its host. These methods could provide a more in-depth examination into how perturbations of the gut microbiome may be influencing host homeostasis.

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## LIST OF ABBREVIATIONS

5HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine or serotonin
ANOVA	Analysis of variance
ASV	Amplicon sequence variants
ATCC	American Type Culture Collection
CNS	Central nervous system
DAT	Dopamine transporter
EC	Enterochromaffin
ENS	Enteric nervous system
GABA	Gamma-aminobutyric acid
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
HPI	Hypothalamic-pituitary-interrenal
HPLC	High-performance liquid-chromatography
HT <sub>1A</sub>	Serotonin 1A receptor
HT <sub>2A</sub>	Serotonin 2A receptor
H <sub>T</sub> PC	Human therapeutic plasma concentration range
LCMS	Liquid chromatography mass spectrometry
LDA	Linear discriminant analysis
LEfSE	Linear discriminant analysis effect size
log K <sub>oc</sub>	Organic carbon normalized sorption coefficient
Log K <sub>ow</sub>	Octanol-water partition coefficient
LeuT	Leucine transporters
MAO-A	Monoamine oxidase type A
MAOI	Monoamine oxidase inhibitors
MIC	Minimum inhibition concentration

MS-222	Tricaine methanesulfonate
NET	Norepinephrine transporter
NGS	Next generation sequencing
OCD	Obsessive compulsive disorders
OECD	Organization for Economic Co-operation and Development
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PERMDISP	Homogeneity of multivariate dispersion
SCFA	Short chain fatty acid
SERT	Serotonin reuptake transporter
SPE	Solid phase extraction
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressants
WWTP	Waste-water treatment plant

## PREFACE

This thesis was prepared in a manuscript style format following the University of Saskatchewan College of Graduate Studies and Research guidelines. Accordingly, chapter 1 of this thesis is a general introduction to the work conducted while chapters 2 and 3 have been prepared for a peer-reviewed scientific journals, and chapter 4 is general discussion pertaining to the above chapters. Therefore, there may be some overlap and redundancy of background information. All references are cited at the end of the thesis while supporting information for the above chapters are within the appendix. The appendix also provides an informative assessment conducted during my master's degree study that is secondary to the thesis, yet relevant to the theme of the work provided here.

## CHAPTER 1: GENERAL INTRODUCTION

## **1.1. Introduction**

The complex microbial network that dominates Earth plays a vital role in the homeostasis of all organisms and their ecosystems. These networks are the building blocks of life on our planet, supporting soil decomposition and nutrient uptake in plants and the ability for organisms to develop, metabolize, and support other physiological and cellular processes (Layeghifard et al., 2017; Tinker, 1984; Van Veen and Kuikman, 1990). Studies involving understanding of how these microbial communities work, specifically understanding endogenous microbes (i.e., the gut microbiome) living within other organisms, have proliferated within the last decade (Goodrich et al., 2014). In regard to the work described here, there continues to be a need to clarify the role of the microbiome in ecotoxicological research, to understand not only how the microbiome is impacted by xenobiotic exposure, but also how a shift in the microbial makeup of such a vital community due to a toxicant can affect host health (Adamovsky et al., 2018).

## **1.2. The Gut Microbiome**

A network of microorganisms coexisting endogenously within a host organism is known as a microbiome (Sekirov et al., 2010). In humans, microbes can be found colonizing all surfaces of the body including the skin and mouth, yet 70% of these microorganisms are located within the gastrointestinal (GI) tract and colon (Rooks and Garrett, 2016; Sekirov et al., 2010). The makeup of the gut microbiome is complex, consisting mainly of bacteria as well as archaea, viruses, fungi and protozoans (Jandhyala et al., 2015; Sekirov et al., 2010). The composition can vary between organisms and among the same species. At times, the composition can fluctuate within an individual as well, yet a set of core microbes have been identified that are markedly consistent within similar species (Rooks and Garrett, 2016).

Sex is an important variable in terms of the gut microbiome composition (Kim et al., 2020). Mammalian, as well as fish studies have recently been conducted determining the distinction of the gut microbiome between the two sexes (DeBofsky et al., 2020; Lozano et al., 2018; Stoffel et al., 2020), realizing that host hormones are key to composition of the microbiome (Chen and Madak-Erdogan, 2016; Markle et al., 2013; Org et al., 2016; Yurkovetskiy et al., 2013). Many studies conducted revealed that females host a more abundant microbiome where alpha diversity was found to be higher (de la Cuesta-Zuluaga et al., 2019; DeBofsky et al., 2020; Li et al., 2016;

Yurkovetskiy et al., 2013). During puberty, the microbiome has been shown to become less abundant in males (Yurkovetskiy et al., 2013). The potential mechanism for this is that androgen receptor activity may influence composition of the microbiome in males (Markle et al., 2013). Some gut microbiota also have the ability to metabolize hormones and influence the expression of host genes relating to sex hormone function (Kunc et al., 2016). There is still a need to investigate why the microbiota composition differs between sexes and the function and connection between hormones and the microbiome. However, with this emerging evidence, it is critical to acknowledge sex within microbiome studies, for sex-specific differences may occur (Kim et al., 2020).

Only recently has it come to light that the community of microbes living within an organism should be considered a functioning organ within the body (Possemiers et al., 2011). This tightly intertwined network can support several essential systems within the body including the immune and nervous systems, and the breakdown, metabolism and synthesis of key nutrients, such as short-chain fatty acids (SCFA) and biogenic amines such as dopamine and serotonin (Lyte and Brown, 2018; Pessione and Cirrincione, 2016; Rhee et al., 2009; Turnbaugh et al., 2007). These microbes not only support their host, the organism they live within, but a symbiotic, bidirectional relationship forms, allowing for cross-talk between host and microbiome. If dysbiosis, or an imbalance of commensal microbiota occur, host homeostasis can be impacted resulting in susceptibility to allergens as well as metabolic and inflammatory conditions (Rooks and Garrett, 2016).

### ***1.2.1. The microbiome-gut-brain-axis***

The enteric nervous system holding more than 500 million neurons can be found embedded in the gastrointestinal lining (Furness, 2006). This system along with the central nervous system, autonomic nervous system, the hypothalamic-pituitary-adrenal (HPA) axis and the brain and spinal cord, create a bidirectional network that allows crosstalk between the gut and brain (Carabotti et al., 2015). Activation of these systems through cytokines, hormones, an amalgam of specialty cells and afferent and efferent signaling, allows for communication between the gut and brain (Butler et al., 2019; Carabotti et al., 2015). It has been shown that microbiota can also influence the interaction between the brain and gut (Emeran A Mayer et al., 2014). This complex is now termed the microbiome-gut-brain-axis, and research on how the



microbiome can influence and communicate with its host has proliferated (Butler et al., 2019). The microbiome is highly influential in terms of stress responses, immunity and the production of SCFA and neurotransmitters (Butler et al., 2019; Macpherson and Harris, 2004; Rooks and Garrett, 2016; Sekirov et al., 2010). Evidence of these abilities come from gnotobiotic (lacking microflora) animals, exhibiting altered responses to stressors due to the lack of bacteria metabolizing vital molecules essential for the development of the enteric nervous system and the HPA axis (Rooks and Garrett, 2016). Noticeable differences in the release of certain molecules or hormones have been identified during stress responses including higher adrenocorticotrophic hormone and corticosterone release during a stress response in gnotobiotic mice (Sudo et al., 2004), as well as a significant reduction in serotonin (Wikoff et al., 2009), specifically during a stressful event (Lyte et al., 2020). These studies shed light on the inability for a more controlled response to stressors when there is a lack of microbiota.

Regarding immunomodulatory effects, gnotobiotic animals again give us insight into the importance of the microbiome on the immune system. Macpherson and Harris (2004) demonstrated that in mice lacking commensal microbiota, the immune system is underdeveloped where numbers of several different types of immune cells including CD4<sup>+</sup> T cells and IgA-producing plasma cells are reduced and the spleen and lymph nodes, where the majority of all immune-cells is made, are lacking structure. Interestingly, such malformations are quickly reversed when these gnotobiotic animals are grouped with others containing microflorae (Macpherson and Harris, 2004). During the development of the immune system, bacteria are critical in the activation of certain receptors eventually causing cytokine production (Butler et al., 2019; Chung et al., 2012).

One of the largest roles microbiota play in the body is the production of SCFAs, which are responsible for a multitude of functions including acting as a source of energy for epithelial cells in the intestine and influencing many molecules that in turn act on metabolism, the immune system, liver and the brain (Butler et al., 2019). Butyrate, a SCFA produced specifically by bacteria, holds a large impact on gene transcription which can eventually influence epigenetic processes (Butler et al., 2019). Studies have attributed butyrate with antidepressant like effects and it ameliorates neurodegeneration in Huntington's, Alzheimer's and Parkinson's disease

models (Ferrante et al., 2003; Govindarajan et al., 2011; Schroeder et al., 2007; Sharma et al., 2015).

Many microbes are also capable of producing neurotransmitters such as gamma-aminobutyric acid (GABA), acetylcholine, dopamine and serotonin. The production of these biogenic amines in microbes are termed “biomediators” rather than neurotransmitters, for their role in microorganisms pertain to intercellular communication, growth and colonization (Roshchina, 2010). Importantly, the production of these molecules by microbiota allows for cross talk between bacteria and host giving insight into how microbes can directly interact with host cells leading to the influence of anxiety, depression, cognition and mood (Butler et al., 2019).

### ***1.2.2. Homologous transporters on bacterial and host cells***

Not only have bacteria been found to produce neurotransmitters, but several express homologous transporter proteins to those of host norepinephrine, dopamine and serotonin transporters (NET, DAT and SERT, respectively) (Androutsellis-Theotokis et al., 2003). These transporters, including the ones found on bacterial cells, are all within the same neurotransmitter:sodium symporter family (Zhou et al., 2009). The bacterial transporters have been mainly identified as amino-acid transporters such as tryptophan transporters from *E. coli* (Androutsellis-Theotokis et al., 2003) and leucine transporters (LeuT) from *Aquifex aeolicus* (Yamashita et al., 2005). Importantly, LeuT has been studied to better understand antidepressant drug abilities to block neurotransmitter reuptake (Zhou et al., 2007). These understandings have helped to identify how antidepressants, such as selective serotonin reuptake inhibitors (SSRIs) bind to SERT, and have fueled the question of whether these pharmaceuticals have the capacity to interact with commensal microbiota and so potentially alter the microbiome (Lyte et al., 2019).

### **1.3. Serotonin and SERT**

Although most notably thought of as a neurotransmitter modulating mood and brain function, 95% of serotonin is produced in the gut (Gershon and Tack, 2007). Serotonin plays an important role in almost all behavioral processes (i.e., pain, sleep, aggression, memory, appetite and sex),

and is critical in the function of the gastrointestinal, cardiovascular, pulmonary and endocrine systems (Berger et al., 2009; Gershon and Tack, 2007).

### ***1.3.1. Serotonin signaling system***

Serotonin or 5-hydroxytryptamine (5-HT) is a monoamine transmitter which can influence neurons in the brain and peripheral nervous system. With its modulatory abilities, when 5-HT binds to one of its 15 known receptors, it can influence a variety of behavioral responses (Berger et al., 2009). The metabolic precursor to serotonin is the essential amino acid, tryptophan, found in dietary protein sources such as meat, seeds, dairy and fruit (Jenkins et al., 2016). While most serotonin is produced within enterochromaffin (EC) cells or neurons within the gut, a small portion is produced in the central nervous system (CNS) (Gershon and Tack, 2007; Jenkins et al., 2016). Tryptophan, once in the CNS, is hydroxylated into 5-hydroxytryptophan and then decarboxylated to 5-hydroxytryptamine, or serotonin (Jenkins et al., 2016). EC cells, being the predominate source of serotonin production within the body, are the main source of blood serotonin for blood platelets that are not capable of producing their own serotonin (Gershon and Tack, 2007). Within the connective tissue of the lamina propria in the GI tract, serotonin is released where it can then act on serotonin receptors. Once serotonin acts upon its receptors within the CNS, the enteric nervous system (ENS), or on epithelial or immune cells, it is then taken up by SERT, which is found on neurons within the serotonergic nerve terminals of the brain, in the enteric nervous system, epithelial cells and on blood platelets. SERT either transports 5-HT back into vesicles or the 5-HT is catabolized by monoamine oxidase type A (MAO-A) into its metabolite; 5-hydroxyindoleacetic acid (5HIAA) (Coates et al., 2017; Gershon and Tack, 2007; Jenkins et al., 2016; Linden et al., 2009; Read and Gwee, 1994). Once in its metabolic form (5HIAA), it is then excreted via urine (Read and Gwee, 1994).

The binding of serotonin to different receptors can cause a variety of modulatory effects. For example, depending on which receptor serotonin binds to within the CNS, it can stimulate sleep, mood, memory or appetite (Berger et al., 2009; Jenkins et al., 2016). Within the GI tract, binding of serotonin to specific receptors can regulate gastric emptying or intestinal peristalsis (Grider, 2003; Raybould et al., 2003). Irritable bowel syndrome has also been shown to be influenced by serotonin (Coates et al., 2017). For example, the binding of serotonin to 5-HT<sub>2A</sub> receptors on smooth muscle cells can stimulate muscle contraction in the gut while binding to 5-

HT<sub>3</sub> receptors can modulate visceral pain transmission (Coates et al., 2017). Serotonin has been found to modulate behaviors similarly within vertebrates including fish, yet for fish, serotonin can also influence gas exchange within gills and intestinal osmoregulation (Kreke and Dietrich, 2008; Morando et al., 2009).

### ***1.3.2. Selective serotonin reuptake inhibitors***

The role of serotonin in the modulation of almost all functions within the body attests to its importance in organism health and function. However, within humans, imbalances of behaviors such as depression, anxiety and obsessive compulsive disorders (OCD) have led to the study of monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs) and eventually SSRIs (Ferguson, 2001). SSRIs demonstrate a selective affinity for serotonin reuptake transporter sites and thus block serotonin from being taken back up into the neuronal vesicles, allowing for prolonged serotonin signaling within the body (Hiemke and Härtter, 2000; Linden et al., 2009). SSRIs have been widely used for treatment of several mood and behavior disfunctions and have been found to be much safer, with milder side effects than MAOIs and TCAs due to target selectivity, solely binding to serotonin receptors (Ferguson, 2001; Hiemke and Härtter, 2000). Fluoxetine, trademarked as Prozac®, was the first SSRI introduced to the United States in 1988 and is currently one of the most widely prescribed SSRIs (Altamura et al., 1994; Ferguson, 2001; Luo et al., 2020).

When orally ingested, the majority of fluoxetine is absorbed and is metabolized into its bioactive primary metabolite, norfluoxetine (Hiemke and Härtter, 2000; Kosjek and Heath, 2010; Silva et al., 2012; Vaswani et al., 2003). Differing from other SSRI metabolites, norfluoxetine retains just as much potency as its parent compound (Kosjek and Heath, 2010). Norfluoxetine is then able to inhibit SERT before it undergoes phase 2 hepatic metabolism, where it is converted into a more hydrophilic compound in order for it to be excreted in urine (Silva et al., 2012a; Vaswani et al., 2003). Norfluoxetine demonstrates a half-life of one to four days and takes on average, in a normal pharmacological regime, four weeks to reach steady state levels within the human body (Altamura et al., 1994; Vaswani et al., 2003). Nonlinear pharmacokinetics have been reported for fluoxetine where at higher doses the plasma concentration is disproportionately higher (Altamura et al., 1994). The majority of fluoxetine, 60 to 75%, is excreted through the kidneys, while 10 to 16% has been found in the bile (Altamura et al., 1994).

## 1.4. Wastewater effluent and SSRIs

With exponential growth in the use of SSRIs worldwide, they are now ubiquitous and commonly detected within aquatic environments (Silva et al., 2015). SSRIs enter wastewater treatment plants (WWTPs) either through domestic waste (i.e., flushing expired/unused pills or human excretion) or from municipal waste from healthcare facilities (Schultz et al., 2011; Silva et al., 2015). Fluoxetine, among the most prevalent SSRIs found downstream of WWTPs, has been detected in waterbodies at ng/L to µg/L (Brooks et al., 2003a; Kolpin et al., 2002; Metcalfe et al., 2010; Silva et al., 2012). Fluoxetine demonstrates an octanol-water partitioning coefficient ( $\log K_{ow}$ ) between 1.22 to 4.0 indicating its relatively low water solubility. It can demonstrate a half-life of around 3 days in water and can adsorb to particulates, soils and sediments with a  $\log K_{oc}$  (organic carbon normalized sorption coefficient) of 3.82 to 5.63 (Black and Armbrust, 2007; Gaworecki and Klaine, 2008; Silva et al., 2015; Velázquez and Nacheva, 2017). Degradation of fluoxetine in water is slow where it does not readily biodegrade by wastewater treatment, it is resistant to photolytic breakdown, and it quickly absorbs to sediments within rivers and streams allowing it to last longer within aquatic habitats (Black and Armbrust, 2007).

### 1.4.1. SSRIs and aquatic ecotoxicology

Serotonin is an evolutionarily conserved molecule found in all vertebrates (McDonald, 2017). For instance, all but one of the serotonin receptors identified in mammals have been found in fish and the binding of SSRIs occurs on the serotonin transporter, SERT, in fish, similar to that of mammalian transporter binding (McDonald, 2017). A few differences in the teleost fish serotonin system compared to mammalian include the fact that serotonin can freely pass through the fish blood brain barrier (BBB) allowing for peripheral serotonin to influence the brain, and instead of enterochromaffin cells that produce the majority of 5-HT in humans, fish have enteric neurons along the intestinal walls that are the main source of this molecule (Anderson et al., 1988; Caamaño-Tubío et al., 2007; Khan and Deschaux, 1997; Velarde et al., 2010). There is still a continued need to study the serotonin system in fish, however, from previous work, serotonin has been identified as a vital part of fish homeostasis (reviewed by McDonald (2017)).

Numerous ecotoxicology studies have been conducted examining the effects of SSRIs on aquatic organisms with major endpoints including physiology, reproduction, and behavior (Silva

et al., 2015). The results from such studies demonstrate reductions in aggression and anxiety (Ansai et al., 2016; Barbosa et al., 2012; Loveland et al., 2014) potentially through the stimulation of the 5-HT<sub>1A</sub> receptor which can lead to a reduced predator-prey responses (McDonald, 2017), appetite suppression (Mennigen et al., 2010) where serotonin is known to inhibit food intake responses by decreasing brain glycogen (McDonald, 2017; Pérez Maceira et al., 2014), and the stimulation of the hypothalamic-pituitary-inter-renal (HPI) and hypothalamic-pituitary-gonadal (HPG) axes leading to modulatory effects on reproductive behavior (Martin et al., 2017; McDonald, 2017). Although such reproductive effects demonstrate some incongruities as reviewed by McDonald (2017). With continued research regarding the effects of SSRIs on fish health, there still are several gaps to be filled where serotonin is known to play important roles including in the GI and cardiovascular systems (McDonald, 2017).

### **1.5. SSRIs and the gut microbiome**

While there is an understanding of the influential role of the gut microbiome on several systems throughout the body including the regulation of serotonin (Fung et al., 2019) and homologous sodium symporters on bacterial and host cells, no studies have been conducted to assess the influence of SSRIs on the gut microbiome of aquatic organisms. In recent years, some studies have identified shifts in the microbial makeup of rodent and human microbiomes due to ingestion of SSRIs as well as studying the antimicrobial properties of SSRIs on commensal bacteria (Ait Chait et al., 2020; Cussotto et al., 2018; Fung et al., 2019; Lyte et al., 2019; Lyte and Brown, 2018; Sun et al., 2019; Zhang et al., 2021). Results include inhibition of growth, *in vitro*, of several dominate commensal microbiota in the human gut (Ait Chait et al., 2020; Cussotto et al., 2018) where some may hold biogenic amine transporters that are known to be inhibited by SSRIs (Fung et al., 2019; Lyte and Brown, 2018). Studies have also found that fluoxetine can induce changes in community structure and diversity of the microbiome of rodents (Fung et al., 2019; Sun et al., 2019) and the susceptibility of microbes associated with a healthy status to antidepressants (Maier et al., 2018). With these recent observations, the importance of the microbiome on host homeostasis, homologous biogenic amine transporters found both on bacterial and eukaryotic cells and recent understandings of the importance of the microbiome in aquatic toxicological studies, it is desirable to better understand the environmental effect of SSRIs, such as fluoxetine, on the gut microbiome of fish.

## **1.6. Characterizing the gut microbiome through 16S rRNA sequencing**

Most commensal bacteria residing within the gut of living organisms are unculturable (Stewart, 2012). Because these microbiota need a very specific environment to survive, and are sensitive to ambient oxygen, it is impractical to use culturing methods to identify the microbial communities that make up the gut microbiome (Browne et al., 2016). Thus, the development and use of next-generation sequencing (NGS) technologies have become important in the discovery of the abundance and diversity of the gut microbiome (Duperron et al., 2020; Walker et al., 2014). Within NGS there are several approaches to advance our knowledge on not only the identification of microbes present using metataxonomics and metagenomics techniques, but how these microbes function and interact with their host through the use of metatranscriptomics, metaproteomics and metabolomics (Whon et al., 2021). For this work, metataxonomic sequencing was conducted to better characterize the gut microbiome of fathead minnow (*Pimephales promelas*) after exposure to fluoxetine. Metataxonomics is a targeted approach unlike metagenomics. It relies on targeting a specific gene region of the 16S rRNA gene found in almost all bacteria as well as some archaeal taxa (Hilton et al., 2016; Walker et al., 2014; Whon et al., 2021). This technique is the most widely used approach in understanding the abundance and types of microbes present in microbiome studies (Hilton et al., 2016). Using the 16S rRNA sequencing technique, the taxonomic classification, structure and composition of the microbiome can be identified (Whon et al., 2021). After DNA extraction, a specific hypervariable region of the 16S rRNA gene is amplified using polymerase chain reaction (PCR) where an amplicon library is created. Once PCR products are purified, the amplicons are ready for sequencing (Kumar et al., 2014). After sequencing of the hypervariable region of the 16S rRNA gene, bioinformatics is performed to cluster sequence reads into amplicon sequence variants (ASVs) based on 97% identity of the reads. Once clustered, phylogenetic relationships, relative abundance and diversity analysis can then be conducted (Kuczynski et al., 2011; Kumar et al., 2014).

## **1.7. Rational**

Gut microbiota hold critical roles in the homeostasis of their host organism. Only recently have studies emerged within ecotoxicology to better understand the influential capabilities these microorganisms have on their host when exposed to an environmental toxicant. The

understanding of the microbiome-gut-brain axis, homologous serotonin transporters on microbial cells, and the antibiotic-like effects antidepressants have on commensal bacteria *in vitro*, give way to the importance of understanding how SSRIs affect the gut microbiome. Due to continued release of pharmaceuticals such as SSRIs into aquatic environments, it is critical to not only look at physiological, behavioral, and molecular responses of organisms to these contaminants, but also to understand how the commensal bacteria, tied to almost all functions of their host, are impacted.

## 1.8. Objectives and Hypotheses

The objectives of this study included:

- 1) Identify if fluoxetine holds antimicrobial properties on bacterial cells, cultured from the gut microbiome of fathead minnows, a commonly studied fish used in ecotoxicological studies.

*H<sub>01</sub>: In vitro, no antimicrobial effects will be found on bacteria cells cultured from the gut microbiome of fathead minnows.*

- 2) Characterize the differences in the gut microbiome of female and male fathead minnows, using 16S rRNA metabarcoding techniques.

*H<sub>02</sub>: No differences in gut microbial makeup will be identified between male and female fish.*

- 3) Identify potential dysbiosis in the gut microbiome of fathead minnows when exposed to select concentrations of fluoxetine during a 28-day period using 16S rRNA metabarcoding.

*H<sub>03</sub>: No alterations in abundance or distribution of the gut microbiome will be identified after fathead minnows are exposed to any concentration of fluoxetine for 28 days.*

- 4) Observe any sex-specific responses of the gut microbiome due to fluoxetine exposure.



*H<sub>04</sub>: No sex-specific responses of the gut microbiome will be identified after exposure to fluoxetine.*

CHAPTER 2: MINIMUM INHIBITORY CONCENTRATION ASSAYS FOR THE  
DETERMINATION OF ANTIMICROBIAL PROPERTIES OF FLUOXETINE ON  
CULTURED BACTERIAL CELLS FROM THE GUT OF FATHEAD MINNOW  
(*PIMEPHALES PROMELAS*)

PREFACE

The main objective of Chapter 2 was to run a preliminary study to determine if fluoxetine holds antimicrobial properties on bacterial cells extracted from the gut of fathead minnows *in-vitro*. The data generated from these assays gave validity to continue running an *in-vivo* study to determine the effects of fluoxetine on the gut microbiome of exposed fathead minnows (Chapter 3). Chapter 2 has been prepared in a manuscript style and is intended to be submitted to the peer-reviewed journal *Scientific Reports*.

Weber, A., DeBofsky, A., Xie, Y., Challis, J., Rubin, J., Giesy, J. (2021). Minimum Inhibitory Concentration Assays for the determination of antimicrobial Properties of Fluoxetine on Cultured Bacterial Cells from the Gut of Fathead Minnow (*Pimephales promelas*).

Author contributions:

Alana Weber (University of Saskatchewan) Designed and performed the assays, analyzed data, prepared figures and tables and drafted the manuscript.

Abigail DeBofsky (University of Saskatchewan) extracted bacterial cells from the gut of fathead minnows and provided edits to the manuscript.

Yuwei Xie (University of Saskatchewan) Provided guidance of design and edits to the manuscript.

Jonathan Challis (University of Saskatchewan) Performed the chemical analysis to measure true concentrations of fluoxetine in the assays and provided edits to the manuscript.

Joseph Rubin (University of Saskatchewan) Assisted with the design and provided equipment and guidance for the assays run. Contributed edits and comments to the manuscript.

John Giesy (University of Saskatchewan) Provided guidance and contributed edits and comments to the manuscript.

## **Abstract**

Toxicants can impact the microbiome of the gut and further influence host health. To better assess if a contaminant holds antimicrobial properties, an *in vitro* assay may be performed on cultured bacterial cells. A minimum inhibitory concentration assay was run to determine if the compound, fluoxetine, a common selective serotonin reuptake inhibitor found in aquatic environments, may impede growth of bacteria cultured from the gut microbiome of fathead minnow (*Pimephales promelas*). Bacterial cells were exposed to serial dilutions of fluoxetine ranging from 0.015 to 128 µg/mL. After overnight incubation, colonies were counted to determine growth of bacteria. At 32, 64 and 128 µg/mL, growth of select fathead minnow bacteria were found to be inhibited. This preliminary data can be used as an indicator that fluoxetine holds some antimicrobial properties and could potentially affect gut microbiota growth and colonization in fathead minnow.

## 2.1. Introduction

The cross-talk between the enteric nervous system, the immune system and the gut microbiome, and the gut microbiome's ability to regulate several physiological functions within its host, gives way to the importance of gut microbiota on overall homeostasis of host organisms and continues to be expounded upon (Carabotti et al., 2015; Cryan and O'Mahony, 2011; Evariste et al., 2019; Rooks and Garrett, 2016). With our knowledge of a microbiome-gut-brain axis and our understanding of the biotransformation abilities of these microbes, it is deemed useful to understand how contaminants, such as antidepressants found in wastewater effluent, may bidirectionally influence the symbiotic relationship between the gut microbial communities and the host organism (Adamovsky et al., 2018; Evariste et al., 2019).

Selective serotonin reuptake inhibitors (SSRI) are common types of antidepressants that bind to serotonin reuptake transporters (SERT) on neurons extending the availability of serotonin within the synaptic space (Altamura et al., 1994). SERT is a member of the neurotransmitter:sodium symporter (NSS) family along with the norepinephrine and dopamine transporters (NET and DAT) (Androutsellis-Theotokis et al., 2003; Zhou et al., 2009). Interestingly, some bacteria also express homologous proteins found in this same family which are used mainly as amino acid transporters (Zhou et al., 2009). One example is the leucine transporter (LeuT) found on *Aquifex aeolicus*. In fact, LeuT has been used to better understand the dynamics behind how SSRIs may bind to neurotransmitter symporters (Yamashita et al., 2005; Zhou et al., 2009). One of the most widely prescribed SSRIs, fluoxetine (Altamura et al., 1994), has been shown to bind to LeuT, inhibiting its transport ability (Zhou et al., 2009). In recent findings, the antimicrobial properties of SSRIs have been determined for several other bacterial species, most likely due to binding affinity of homologous transporters (Bohnert et al., 2011; Lyte and Brown, 2018; Munoz-Bellido et al., 2000). Due to bacterial inhibitory properties of SSRIs, it is important to determine whether these antidepressants can exert an effect on the gut bacteria of a host that willingly consumes SSRIs and on organisms that may be involuntarily exposed to them due to polluted waters and habitats.

SSRIs have been detected downstream of wastewater treatment plants and in surface waters (Kolpin et al., 2002; Metcalfe et al., 2010; Silva et al., 2012). A multitude of studies have been done to understand how SSRIs, such as fluoxetine, may affect aquatic organisms (ibid Chapter 1;

(Brooks et al., 2003b; Foran et al., 2004; Lister et al., 2009; McDonald, 2017; Painter et al., 2009; Polverino et al., 2021). However, no study has yet been conducted to better understand if fluoxetine can indeed perturb bacteria living commensally in the gut of aquatic organisms and how this may affect the long-term homeostasis of the host.

To better understand if fluoxetine can impact gut microbiota of a commonly studied fish species, fathead minnow (*Pimephales promelas*), an *in vitro* bacterial cell study was first conducted. Minimum inhibitory concentration (MIC) assays were performed to identify if fluoxetine can impede bacterial growth. Five dominant bacterial species cultured from the gut microbiome of fathead minnows as well as several bacterial cells used in conventional MIC testing, were exposed to serial dilutions of fluoxetine ranging from 0.015 µg/mL to 128 µg/mL. After overnight incubation, colonies were counted to identify growth at each concentration.

## **2.2. Materials and methods**

### **2.2.1. Experimental design**

Minimum inhibitory concentration (MIC) assays were performed to identify whether fluoxetine can inhibit growth of several strains of bacteria, including those cultured from the gut of fathead minnows. The MIC method is defined as being the lowest concentration of the presumed antimicrobial agent that inhibits visible growth of the bacteria or microorganisms within a specific time-frame (EUCAST and ESCMID, 2003). Bacteria were cultured from the gut of fathead minnows by removing fish intestines, which were then homogenized and plated at serial dilutions of  $10^{-6}$ ,  $10^{-5}$ ,  $10^{-4}$  using three different medias including blood, nutrient, and MacConkey. Plates were incubated at 37°C until growth occurred. Growths were differentiated then plated separately and incubated again. Once culture isolation was achieved, bacteria were sent for sequencing using the cpn60 primers (Fernando and Hill, 2021). When bacteria were identified and sequenced they were stored at -80 °C until further use. Cultured bacteria consisted of *Aeromonas veronii* AMC 35, *Plesiomonas shigellides* 302-73, *Aeromonas veronii* Hm21, *Staphylococcus hominis* subsp. *novobiose* and *Aeromonas ichthiosmia* CECT 4489. Four American Type Culture Collection (ATCC) bacteria commonly used in MIC assays were also tested, including, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Five *E. coli* strains cultured from wild birds were also included in this study, consisting of *E. coli* 136C, 99A, 107A, 112A, 97A. Lastly, two sensitive bacterial species

were also tested: *Bacillus subtilis* and *Micrococcus luteus*. Bacteria were removed from frozen storage and were streaked on Mueller-Hinton agar-based plates (Millipore Sigma, Germany). Plated bacteria were left to incubate overnight at 35 °C. After overnight incubation, three to five colonies were swabbed and placed in sterile McFarland tubes and turbidity was measured. Two-fold serial dilution between 0.015 and 128 µg/mL of fluoxetine (TCI America, Oregon) were added to 18mL of sterilized Mueller Hinton agar (Table 2.1). Fluoxetine concentrations used were as follows: 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.125, 0.06, 0.03, and 0.015 µg/mL. The fluoxetine-agar solution was then poured onto petri dishes and allowed to set. Bacteria standardized to McFarland scale of 0.5 were then plated on the fluoxetine-agar petri dishes using a 48-prong agar stamper. Pins on the agar stamper were dipped into each bacterial suspension and pressed into the fluoxetine-agar plates. Plates were then incubated in 35 °C for 24 hours. MIC detection was performed by checking for growth of bacteria and compared to control plates (Image 2.1). Testing was performed in duplicate and MIC results were analyzed by observing at what concentration of fluoxetine was growth of bacteria inhibited.

### **2.2.2. Fluoxetine quantification**

To confirm fluoxetine concentrations, all dilutions used in MIC testing were sub-sampled (1 mL) into vials and spiked with fluoxetine-d5 at a target concentration of 50 µg/L. Fluoxetine concentrations were then confirmed using a Vanquish UHPLC and Q-Exactive™ HF Quadrupole-Orbitrap™ mass spectrometer (Thermo-Fisher). Liquid chromatography (LC) separation was carried out on a Kinetex 1.7µm Biphenyl LC column (100 x 2.1 mm) (Phenomenex, Torrance, CA) using an isocratic elution of 40% H<sub>2</sub>O: 60% acetonitrile (Fisher Scientific) at a flow rate of 0.2 mL/min and column temperature of 40°C. Fluoxetine and fluoxetine-d5 had a retention time of 1.5 min. Detailed liquid chromatography tandem mass spectrometry (LC-MS) parameters can be found in Appendix A. Along with fluoxetine quantification, percent error was calculated to identify the difference between the nominal concentration and the measured. Percent error was calculated by subtracting the measured concentration by the nominal concentration and dividing by the nominal concentration.

### 2.3. Results

The lowest measured concentrations were found to hold greater percent error compared to the higher nominal and measured concentrations (Table 2.2).

**Table 2.1** | Nominal and measured concentrations of fluoxetine in agar used for MIC assays. The greater the nominal concentration the less percent error was observed.

Nominal concentrations (µg/mL)	LC-MS measured concentrations (µg/mL)	Percent error
0.015	0.0064	57.65%
0.03125	0.0087	72.29%
0.0625	0.0478	23.51%
0.125	0.1023	18.17%
0.25	0.1984	20.63%
0.5	0.4208	15.84%
1	0.9549	4.51%
2	1.9941	0.30%
4	3.9193	2.02%
8	7.9063	1.17%
16	15.6922	1.92%
32	31.2306	2.40%
64	63.3688	0.99%
128	Not measured*	N/A

\* There was not enough agar left in the 128 µg/mL solution to perform the analytical analysis, however, by observing the other greater concentrations we can be certain a measured concentration would not be more than a few percentage points off from the nominal concentration.

The MIC assays resulted in inhibition of growth for several bacteria at the greater concentrations of fluoxetine in agar (Table 2.3). All replicates besides Wild Bird *E. coli* 136C and 112A were found to be inhibited at the same concentrations. The minimum inhibitory concentrations of fluoxetine for the five bacteria cultured from the gut microbiome of fathead minnow were as follows: for *Aeromonas veronii* AMC 35, *Aeromonas veronii* Hm21 and *Staphylococcus hominis* subsp. *novobiose* the MIC was 32 µg/mL; For *Plesiomonas shigelloides* 302-73 and *Aeromonas ichthiosmia* CECT 4489, the MIC was 64 µg/mL.

As for ATCC bacteria, *Staphylococcus aureus* and *Enterococcus faecalis* held MICs of 64 µg/mL and MIC for *Escherichia coli* was 128 µg/mL. Fluoxetine was ineffective against the ATCC *Pseudomonas aeruginosa* due to its ability to grow even at the highest concentration of

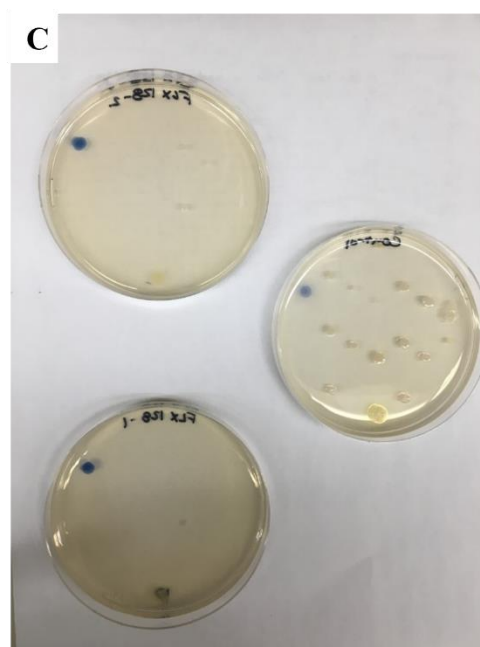
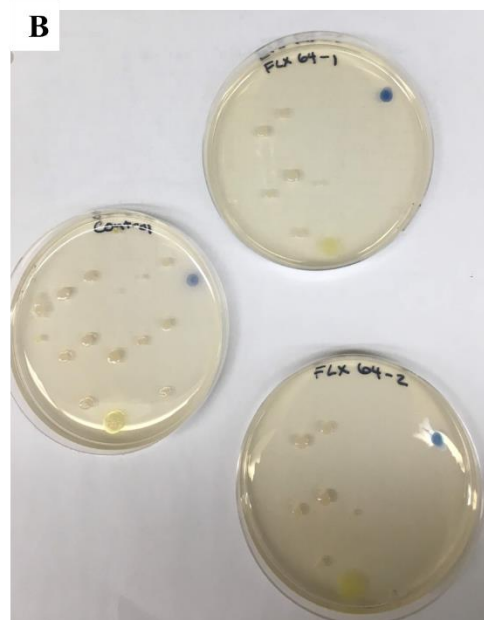
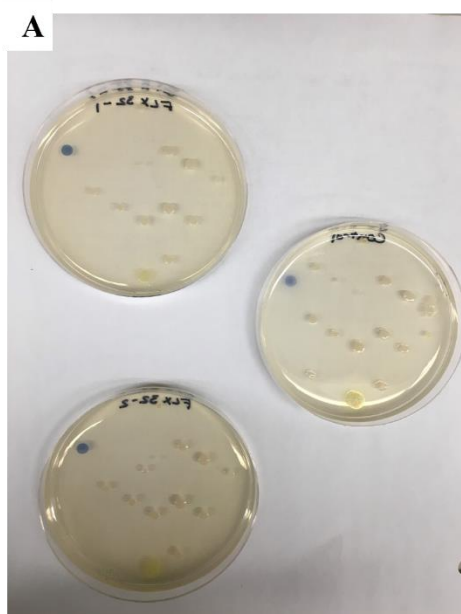


the compound. Wild bird *E. coli* 136C, 99A, and 112A were not affected by fluoxetine even at the highest concentrations, however, strains 97A and 107A were inhibited at 128 µg/mL. For the two sensitive bacteria, *Bacillus subtilis* and *Micrococcus luteus*, the MICs were 64 and 32 µg/mL, respectively.

**Table 2.2** | MIC results depicting measured fluoxetine concentrations where inhibition of bacterial growth occurred. Asterisks represent the five strains cultured from the gut microbiome of fathead minnows. NI represents “No Inhibition” of growth. Because true concentrations were not measured for the nominal 128 µg/mL fluoxetine-agar mixture, ~128 is shown here. Wild Bird *E. coli* 136C and 112A were found to be inhibited at 128 µg/mL in replicate 1 but not replicate 2 plates.

Bacteria strain	LC-MS measured concentration of fluoxetine (µg/mL)
<i>Aeromonas veronii</i> AMC35*	31.23
<i>Plesiomonas shigelliodes</i> 302-73*	63.37
<i>Aeromonas veronii</i> Hm21*	31.23
<i>Staphylococcus hominis</i> subsp. <i>novobiuse</i> *	31.23
<i>Aeromonas ichthiosmia</i> CECT 4489*	63.37
ATCC <i>Staphylococcus aureus</i>	63.37
ATCC <i>Enterococcus faecalis</i>	63.37
ATCC <i>Escherichia coli</i>	~128
ATCC <i>Pseudomonas aeruginosa</i>	NI
Wild bird <i>E. coli</i> 136C	~128/NI
Wild bird <i>E. coli</i> 99A	NI
Wild bird <i>E. coli</i> 107A	~128
Wild bird <i>E. coli</i> 112A	~128/NI
Wild bird <i>E. coli</i> 97A	~128
<i>Bacillus subtilis</i>	63.37
<i>Micrococcus luteus</i>	31.23

All bacteria cultured from the gut of fathead minnows were found to be inhibited by fluoxetine. Fluoxetine also demonstrated inhibitory effects on ATTC bacteria except for *Pseudomonas aeruginosa*. As for wild bird *E. coli* strains, only two of five were found to be inhibited. The two sensitive bacteria used in standard MIC testing, *Bacillus subtilis* and *Micrococcus luteus* were found to be inhibited at ranges similar to that of the bacteria from fathead minnows.



**Image 2.1** Images of fluoxetine inoculated petri-dishes and bacteria growth. (A) depicts duplicates of 32  $\mu\text{g/L}$  of fluoxetine dishes next to the control, (B) 64  $\mu\text{g/L}$  fluoxetine dishes and control, and (C) shows the 128  $\mu\text{g/L}$  fluoxetine dishes and control. Controls are shown on the right side in photographs A and C and on the left side in photograph B.

## 2.4. Discussion

A minimum inhibitory concentration assay was conducted to assess if fluoxetine can impede growth of bacteria cultured from the gut microbiome of a commonly studied fish species, *P. promelas*. After a 24-hour incubation period of bacterial cells inoculated in two-fold serial dilutions between 0.015 and 128  $\mu\text{g/mL}$  of fluoxetine-Mueller Hinton broth solution, all bacteria from the gut of fathead minnows were found to be inhibited by concentrations of fluoxetine between 32 and 64  $\mu\text{g/mL}$  of fluoxetine. Several other bacterial strains included in this study that have been used for standard MIC testing as well as ATCC bacteria were also found to be inhibited by fluoxetine at similar concentrations.

It is important to understand that the fluoxetine concentrations used in the MIC assays are not environmentally relevant where most studies have found fluoxetine to be between ng/L to low  $\mu\text{g/L}$  in waterbodies. However, in the context of MIC assays to determine antimicrobial like properties, greater concentrations are needed of the compound, typically at  $\mu\text{g/mL}$ , in order to identify bacteriostatic and bactericidal effects (Lyte et al., 2019).

SSRIs have exhibited antimicrobial properties such as their ability to inhibit slime synthesis, swarming and cellular growth (Munoz-Bellido et al., 2000). Previous studies discovered similar results as found here including inhibition of growth of *Escherichia coli* and *Staphylococcus aureus* due to fluoxetine exposures (Batista de Andrade Neto et al., 2019; Cussotto et al., 2018; Jin et al., 2018). Batista de Andrade Neto et al., 2019 found fluoxetine to impede growth of *S. aureus* at similar concentrations as what was found in this study, while Cussotto et al., 2018 found growth inhibition of *E. coli* at concentrations much greater than identified here. Munoz-Bellido et al. (2000), found *E. coli*, *S. aureus*, and *E. faecalis* all to be inhibited at lower concentrations than were found in this study, when looking at the SSRI, sertraline.

Speculations of the mechanisms behind SSRIs' ability to impede growth of bacteria include inhibition of efflux pumps (Munoz-Bellido et al., 2000), which are pumps found on bacterial cells that extrude toxicants from the cell (Webber and Piddock, 2003), as well as inhibition of sodium symporters that are homologues to the serotonin reuptake transporter expressed on neuron and non-neuronal cells (Lyte and Brown, 2018). Lyte and Brown, 2018, identified two important biogenic amine transporters on a species of *Lactobacillus* which fluoxetine can block, suggesting a competitive interaction for this transporter and in-turn a potential to perturb

bacterial homeostasis (Lyte and Brown, 2018). The discrepancies between inhibition of similar bacteria as described previously, could be species as well as strain specific (Lyte and Brown, 2018), where the expression of proteins being inhibited may be found on only certain strains of the same species. For instance, two species within the same genus of *Lactobacillus* were found to differentially express biogenic amine transporters where *L. salivarius* expressed the transporters while *L. rhamnosus* did not (Lyte and Brown, 2018). In the current study, all bacteria cells cultured from the gut of fathead minnows were found to be inhibited by fluoxetine at lower concentrations than most ATCC cultures as well as *E. coli* strains from wild birds. This difference could potentially be due to fish gut microbiota being more sensitive to compounds with antibacterial properties.

Based on the possibility of SSRIs impeding bacterial growth, we wanted to identify if the bacteria found in the gut microbiome of a standard aquatic toxicological studied organism, the fathead minnow, could be inhibited *in vitro*. This was a preliminary study conducted to enhance the understanding of how, *in-vivo*, gut bacteria may be perturbed when their host is exposed to fluoxetine. Because of the influential roles the gut microbiome has on its host, including helping with digestion, the ability to signal to the brain and immune system, and its detoxification mechanisms (Butler et al., 2019; Cryan and O'Mahony, 2011; Koppel et al., 2017), it is critical to understand how this network of microbiota is impacted when organisms, importantly for this work, fish, are exposed to environmental pollutants such as the pharmaceutical, fluoxetine.

The results of this study suggest that there is a potential for fluoxetine to impede bacterial growth *in vitro*, which helps support the need to conduct an *in vivo* study to better assess how the gut microbiome may be perturbed when the host is exposed to this SSRI.

## CHAPTER 3: EFFECTS OF AQUEOUS FLUOXETINE ON THE GUT MICROBIOME OF ADULT FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

### PREFACE

The main objective of Chapter 3 was to assess the effect of fluoxetine on the gut microbial composition of fathead minnows at three different concentrations. With the understanding from Chapter 2 that fluoxetine can impede growth of select bacteria cultured from the gut of fathead minnow, it is important to understand if, *in vivo*, fluoxetine continues to hold antimicrobial like properties on the gut microbiome. By measuring alpha and beta diversity, comparing the relative abundance of bacteria at each concentration and exploring a network analysis associated with the influence of fluoxetine on the bacterial communities, we found that at the highest concentration of fluoxetine that fathead minnows were exposed to, held significant ramifications to the gut microbiome. It was also identified that the sex of the host plays an important role in the microbial makeup of the gut and female and male gut microbiomes were differentially affected by fluoxetine. Chapter 3 was prepared in a manuscript style where it will be submitted to the peer-reviewed journal *Science of the Total Environment*.

Weber, A., Xie, Y., Challis, J., DeBofsky, A., Ankley, P., Hecker, M., Jones, P., Giesy, J. (2021) Effects of Aqueous Fluoxetine Exposure on the Gut Microbiome of Adult Fathead Minnow (*Pimephales promelas*). *Science of the Total Environment*.

#### Author Contributions:

Alana Weber (University of Saskatchewan) Designed and performed the fish exposure. Performed all tissue extractions, ran DNA extraction, amplification, purification and sequencing. Conducted bioinformatics and data analysis, generated tables and figures, and drafted the manuscript.

Yuwei Xie (University of Saskatchewan) Helped with design and implementation of the fish exposure. Contributed with fish tissue extractions, DNA amplification, purification and sequencing. Helped with bioinformatics, data analysis, the generation of figures and provided edits and comments for the manuscript.

Jonathan Challis (University of Saskatchewan) Helped with the fish exposure, fish dissections and ran the chemistry analysis on water samples. Provided feedback and edits on the manuscript.

Abigail DeBofsky (University of Saskatchewan) Helped with design and implementation of the fish exposure and fish dissections. Gave insight into DNA amplification, purification and sequencing and helped with bioinformatics and data analysis. Provided feedback on the manuscript.

Phillip Ankley (University of Saskatchewan) Helped with the fish exposure, fish dissections, and DNA sequencing. Gave insight into bioinformatic techniques and provided feedback on the manuscript.

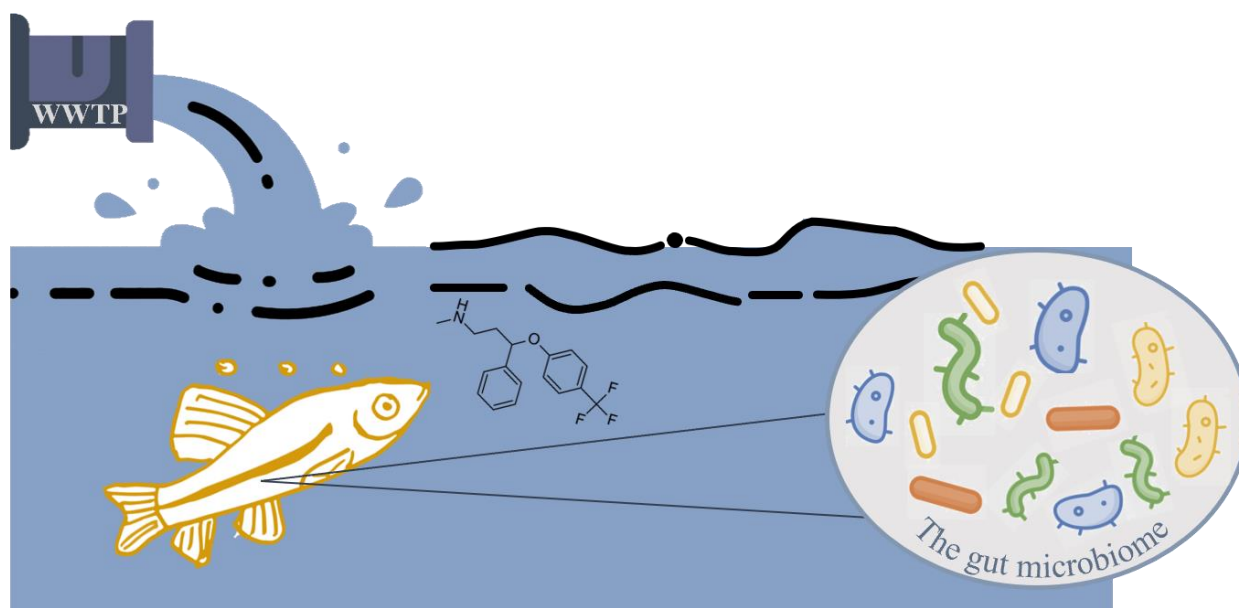
Markus Hecker (University of Saskatchewan) Provided equipment and guidance for the fish exposure.

Paul Jones (University of Saskatchewan) Provided equipment for the chemical analysis.

John Giesy (University of Saskatchewan) Provided guidance of design, implementation and equipment for the fish exposure. Gave feedback and edits on the manuscript.

## Abstract

The microbiome of the gut is vital for homeostasis of hosts with its ability to detoxify and activate toxicants, as well as signal to the immune and nervous systems. However, in the field of environmental toxicology, the gut microbiome has only recently been identified as a measurable indicator for exposure to environmental pollutants. Antidepressants found in effluents of wastewater treatment plants and surface waters have been shown to exhibit antibacterial-like properties *in vitro*, where some bacteria are known to express proteins homologous to those that bind antidepressants in vertebrates. Therefore, it has been hypothesized that exposure to antidepressant drugs might affect gut microbiota of aquatic organisms. In this study, the common antidepressant, fluoxetine, was investigated to determine whether it could modulate the gut microbiome of adult fathead minnows. A 28-day, sub-chronic, static renewal exposure was performed with nominal fluoxetine concentrations of 0.01, 10 or 100 µg/L. Using 16S rRNA amplicon sequencing, shifts among the gut-associated microbiota were observed in individuals exposed to the highest concentration, with greater effects observed in females than in males. These changes were associated with a decrease in relative proportions of commensal bacteria, which can be important for the health of fish including bacteria essential for fatty acid oxidation, and an increase in relative proportions of pathogenic bacteria associated with inflammation. Results demonstrate, for the first time, how antidepressants found in some aquatic environments may influence the gut microbiota of fishes.



**Illustration 3.1**| Graphical abstract representing the gut microbial composition of fathead minnows when exposed to fluoxetine.



### 3.1. Introduction

The vertebrate gut microbiome contributes to the development and function of the nervous system (Sampson and Mazmanian, 2015; Sharon et al., 2016). Thought of as the second brain, the enteric nervous system, embedded in the gastrointestinal lining, holds over 500 million neurons (Furness, 2006). These gastrointestinal neurons allow for communication between the host and gut microbiota (Carabotti et al., 2015; Forsythe and Kunze, 2012; Sampson and Mazmanian, 2015). Within the microbiome-gut-brain axis, gut microbiota play important roles in homeostasis of vertebrate hosts, for instance, regulating gastrointestinal functions, immune system function, and modulation of anxiolytic behavior, perception of pain and emotions (Amaral et al., 2008; E. A. Mayer et al., 2014; Rhee et al., 2009; Sharon et al., 2016). Some neurological conditions, such as Alzheimer's disease, Parkinson's disease, autism and depression, can be, at least in part, attributed to dysbiosis or deregulation of the gut microbiome (Harach et al., 2017; Kim et al., 2018; Naseribafrouei et al., 2014; Strati et al., 2017). The majority of such research has focused on mammals, while studies of the relationship between gut microbiomes of fishes and neurological events are still lacking (Butt and Volkoff, 2019). However, it has been found that the gut microbiome can influence the hypothalamic-pituitary-inter-renal axis (HPI) as well as the stress response in fish, which in turn can affect immune system function, feeding behaviors and overall homeostasis (Butt and Volkoff, 2019; Davis et al., 2016).

Microbiomes in guts of animals can influence major neurotransmitters. The essential neurotransmitter, serotonin (5-hydroxy-tryptamine, 5-HT), can be regulated by certain enteric microbes (*Turcibacter sanguinis* and *Lactobacillus salivarius*) perhaps stimulating host 5-HT biosynthesis (Fung et al., 2019; Lyte and Brown, 2018; Yano et al., 2015). Recently, several studies have assessed how Selective Serotonin Reuptake Inhibitors (SSRIs) as well as other psychoactive drugs affect gut microbiota of rodents with the aim to elucidate possible effects on humans. Findings include changes in the absolute and relative gut microbiome composition and antimicrobial-like properties of fluoxetine on certain bacteria including *Turcibacter sanguinis*, *Lactobacillus rhamnosus*, *Escherichia coli* as well as the family *Peptostreptococcaceae* (Cussotto et al., 2018; Fung et al., 2019; Lyte et al., 2019; Sun et al., 2019; Zhang et al., 2021).

Consumption of antidepressants is continuously increasing worldwide (OECD, 2017) and these compounds are now commonly observed in wastewater effluents and downstream aquatic environments at concentrations of nanograms to micrograms per liter (Ding et al., 2017; Kolpin et al., 2002; Kreke and Dietrich, 2008; Schultz et al., 2011). Among prescription antidepressants, fluoxetine is a model compound for studying the toxicological effects of antidepressants. Fluoxetine (under the trade name Prozac<sup>®</sup>) is one of the most prominently used SSRIs (Luo et al., 2020). After transformation in the body, less than 10% of fluoxetine is excreted in its unchanged, more lipophilic state, while 20% is excreted as its primary metabolite, norfluoxetine (Silva et al., 2012). Concentrations of fluoxetine have been found at 0.012 µg/L in freshwater streams and 0.09 µg/L in wastewater effluent in North America (Brooks et al., 2003a; Kolpin et al., 2002; Metcalfe et al., 2010; Silva et al., 2012a). Within raw sewage concentrations of 0.01 to 3.5 µg/L have been reported (Ding et al., 2017). Fluoxetine has also been detected in tissues of wild fish between 0.02 and 1.58 ng/g (Brooks et al., 2005; Chu and Metcalfe, 2007; Schultz et al., 2010). Bioconcentration of fluoxetine in the Japanese medaka (*Oryzias latipes*) (Paterson and Metcalfe, 2008) and *Daphnia magna* (Ding et al., 2017) leads to concerns for ecotoxicological risks to aquatic animals.

There have been multiple studies focusing on toxicological endpoints of SSRIs on aquatic organisms including reproduction, physiology, and behavior. Findings suggest reductions in behaviors related to aggression and anxiety, modulating the predator-prey response, suppression of appetite, and stimulation of the hypothalamic-pituitary-gonadal (HPG) and the HPI axes contributing to reproduction impairments of fish (Brooks et al., 2003a; McDonald, 2017; Polverino et al., 2021). Until now, no research has been conducted to determine effects of SSRIs on the gut microbiome of fishes.

To investigate how fluoxetine might modulate the microbiome of the gut of the fathead minnow (*Pimephales promelas*), a sub-chronic aqueous exposure of fluoxetine ranging from an environmentally relevant concentration, 0.01 µg/L, to a sub-lethal concentration of 100 µg/L was conducted. The objectives of this study were to: (1) Identify potential differences in the gut microbiome of male and female fathead minnows; (2) Detect effects of fluoxetine on the abundance and diversity of the gut microbiome of fathead minnows; and (3) Identify any sex-specific responses of the gut microbiome due to exposure to fluoxetine.

## 3.2. Materials and methods

### 3.2.1. *Fish husbandry, aqueous exposure and dissection*

Adult fathead minnows, approximately 6 months old, were obtained from the Aquatic Toxicology Research Facility at the University of Saskatchewan. A density of five fish, both males and females, were randomly assigned to a 20-L tank and tanks were randomly assigned a fluoxetine concentration ( $n = 25$  per group, 5 fish per tank, 5 tanks per group, 4 groups). Tanks were aerated using air pumps and dechlorinated water was sourced from the municipal Saskatoon water supply. Fluoxetine hydrochloride (ThermoFisher Scientific, Waltham, MA) was dissolved in water to make a stock solution from which working solutions were then made. Nominal exposure concentrations consisted of a control group (0  $\mu\text{g/L}$ ), low concentration group (0.01  $\mu\text{g/L}$ ), middle concentration group (10  $\mu\text{g/L}$ ), and high concentration group (100  $\mu\text{g/L}$ ). After one week of acclimation, fluoxetine-treated water was added to each tank. Fish were fed twice daily with 2 mg of blood worms and water temperatures were consistently monitored (average = 22 °C  $\pm$  0.7). Ammonia, pH, nitrites, nitrates and dissolved oxygen were monitored in each tank and light to dark ratio was 16:8 according to OECD 229 guidelines (OECD, 2009). This was a static renewal test where tanks were siphoned daily where two-thirds of the tank solution was replaced with freshly prepared fluoxetine treated water. Water samples were taken twice daily, before and after water renewal, for analysis of fluoxetine concentrations.

After 28 days, fish were anesthetized in MS-222 and mass and length were measured. Fish were then euthanized by decapitation and whole gut including tissue and gut contents were collected using sterile techniques. When possible, sex of fish was recorded. All samples were placed in sterile cryovials and held in liquid nitrogen before being placed in a -80 °C freezer until analyses. Maintenance of fish was in line with the animal use protocols (Protocol #20090108) approved by the Animal Research Ethics Board at the University of Saskatchewan.

Overall fish health was determined following Fulton's condition factor (K) calculated from the mass and length of the fish (Equation 1) (Carlander, 1969).

$$K = \frac{\text{Mass}}{\text{Length}^3} \times 100 \quad (1)$$

### **3.2.2. Quantification of aqueous fluoxetine**

Stock solutions of fluoxetine and fluoxetine-d5 (ThermoFisher Scientific, Waltham, MA) were made in HPLC grade methanol (Fisher Scientific) at 100 mg/L. A seven-point calibration curve ranging from 0.5 to 500 µg/L and spiked with 50 µg/L fluoxetine-d5 was used for quantification by isotope dilution (linearity > 0.999 for all analyses). The 10 and 100 µg/L exposure solutions were sub-sampled (1 mL) directly into LC vials and spiked with fluoxetine-d5 at a target concentration of 50 µg/L for analysis. Control and 0.01 µg/L exposure solutions were sub-sampled (40 mL) into 45 mL falcon tubes, spiked with fluoxetine-d5, and extracted using solid phase extraction (SPE) OASIS™ HLB cartridges (6cc, Waters Corporation, Milford, MA). After pre-conditioning with methanol followed by water, 40 mL of water samples were drawn through at approximately 5 mL min<sup>-1</sup>, cartridges were then vacuum dried, and eluted with 2 × 3 mL fractions of methanol (combined). Extracts were evaporated to dryness under nitrogen in a water bath at 40 °C, and reconstituted in 0.5 mL of 50:50 MeOH-H<sub>2</sub>O into amber LC vials. Fluoxetine was quantified using a Vanquish UHPLC and Q-Exactive™ HF Quadrupole-Orbitrap™ mass spectrometer (Thermo-Fisher). LC separation was achieved with a Kinetex 1.7 µm Biphenyl LC column (100 x 2.1 mm) (Phenomenex, Torrance, CA) by gradient elution with 95% water + 5% methanol (A) and 100% methanol (B), both containing 0.1% formic acid at a flow rate of 0.2 mL min<sup>-1</sup> and column temperature of 40 °C. The gradient method started at 10%B, ramping linearly to 100%B over 7 min, held for 1.5 min, and returning to starting conditions for column re-equilibration between 8.5 to 11 min. Further details on LC-MS parameters can be found in Appendix A. All samples were analyzed for the 100 and the 10 µg/L exposure groups while a subset (*n*=11, per group, Table 3.1) of the samples were analyzed for the 0.01 µg/L and control samples using SPE techniques.

### **3.2.3. Extraction of Bacterial DNA and 16S rRNA metagenomics**

DNA was extracted from whole intestines using the DNeasy PowerSoil Kit (Qiagen Inc., Mississauga, ON). Concentrations of DNA were measured using a Qubit 4 Fluorometer and dsDNA HS assay kit (ThermoFisher Scientific, Waltham, MA). The V3-V4 hypervariable region of the bacterial 16S ribosomal RNA gene was amplified by polymerase chain reaction (PCR) with dual-tagged primers, 341F (5'-tag- CCTACGGGNGGCWGCAG-3') and 806R (5'-tag-

GGACTACNVGGGTWTCTAAT-3'). PCR products were checked by agarose gel electrophoresis and no bands were observed for blank controls. Products were then purified using the QIAquick 96 PCR Purification (Qiagen Inc., Mississauga, ON) following manufactures instructions. Library preparation and next generation sequencing (NGS) was conducted following procedures previously published by DeBofsky et al. (2020). Briefly, The NEBNext® DNA Library Prep Master Mix Set for Illumina® (New England BioLabs Inc., Whitby, ON) was used for library construction, and prior to sequencing, quantification of samples was conducted using the Library Quant kit for Illumina®. Sequencing was run on an Illumina® MiSeq sequencer (Illumina, San Diego, CA).

#### **3.2.4. 16S rRNA metabarcoding and bioinformatics**

Bioinformatics analyses were conducted under the QIIME2 v. 2020.10 environment (Bolyen et al., 2019). Adapters were trimmed using Trimmomatic (Bolger et al., 2014). After demultiplexing, amplicon sequence variants (ASV) were denoised and extracted using DADA2 (Callahan et al., 2016) where low quality and primer regions were removed by truncating forward reads at 260bp, and reverse reads at 220bp. Taxonomy was assigned using the feature classifier, VSEARCH (Rognes et al., 2016), against SILVA 132 reference database (Bokulich et al., 2018; Quast et al., 2013). All unassigned and nonbacterial ASVs were then removed, and alpha rarefaction was performed where a sampling depth of 11,125 was decided based on the ability to maximize the depth threshold while minimizing sample loss (Estaki et al., 2020). In total, 73 samples were retained after rarefaction including 31 males and 42 females. Of the males, there were 8 in the control group, 8 in the low group, 6 in the middle group and 9 in the high group. Of the females there were 11 within the control group, 11 in the low group, 9 in the middle group and 11 in the high group.

#### **3.3. Statistics**

All statistical analysis were conducted in R Statistical Language v 4.0.3 (R Core Team, 2020). Normal distribution and equal variance were first determined using a quantile-quantile plot (Q-Q plot), residuals vs fitted plots, Shapiro-Wilk test and a Levene's test (Borcard et al., 2011). To determine fitness of fish, sexes were separated, and K was identified (equation 1) for each fish. If the assumption of a normal distribution was met, an analysis of variance (ANOVA) was used to test condition factor to exposure concentrations of fluoxetine as well as tank number.

If normal distribution parameters were not met, data was log transformed before running a Levene's test to determine equal variance and an ANOVA was then performed. Alpha diversity, beta diversity and relative and differential abundance of ASVs were analyzed with Phyloseq v 1.32.0 (McMurdie and Holmes, 2013).

For parametric testing, a two-sided Student's t-test was applied when evaluating differences between sexes and an ANOVA with a Tukey's HSD was performed to evaluate differences between exposure groups. If assumptions of equal variance were not met while still being normally distributed, a Welch's t-test was run for sex differences. The non-parametric tests performed when normality was not met included a Wilcoxon rank sums test used to compare differences in alpha diversity between sexes and a Kruskal-Wallis test with a Dunn's post-hoc test used for alpha diversity between exposure groups. To compare beta diversity metrics for both sex and exposure group, a multivariate analysis of variance with permutation (PERMANOVA) was conducted along with testing for homogeneity of multivariate dispersion (PERMDSIP) (Anderson, 2001; Dalgaard, 2008; McArdle and Anderson, 2001; Xia et al., 2018). Because sex was found to explain portions of alpha and beta diversity, analyses comparing exposure groups were conducted by separating the sexes. To determine differential abundance of taxa, a linear discriminant analysis (LDA) effect size (LEfSe) was performed (Segata et al., 2011). Neighborhood selection relationships between ASVs (Meinshausen and Bühlmann, 2006) were constructed by the SPIEC-EASI package (Kurtz et al., 2015). Correlations between relative abundance of ASV and aqueous concentration of fluoxetine were confirmed to be robust if the adjusted false discovery rate (FDR) was statistically significant ( $P_{\text{FDR}} < 0.05$ ). The network was displayed and analyzed with Cytoscape V3 (Otasek et al., 2019).

### **3.4. Results**

#### ***3.4.1. Aqueous concentration of fluoxetine and fitness of Fish***

Daily, mean aqueous concentrations after each water change and 24 hours after each renewal were measured (Table 3.1). Measurements of fluoxetine in water confirmed that concentrations consistently decreased between renewal periods (24 hr). Mean (SD) pH of water was  $7.9 \pm 0.2$  throughout the exposure. Condition factors of both female (Appendix B.A, ANOVA test,  $p > 0.05$ ;  $n = 48$ ) and male (Appendix B.B, ANOVA test,  $p > 0.05$ ;  $n = 32$ ) fish were not

significantly affected by exposure to fluoxetine. There were two mortalities during the fish exposure, however, not attributable to fluoxetine itself.

**Table 3.1** | Mean and standard deviation (SD) of aqueous concentrations of fluoxetine right after each water-change (1<sup>st</sup> sampling), 24 hours after renewals (2<sup>nd</sup> sampling) and predicted plasma concentration based on the fish plasma model and linear equation model identified by Margiotta-Casaluci et al. (2014).

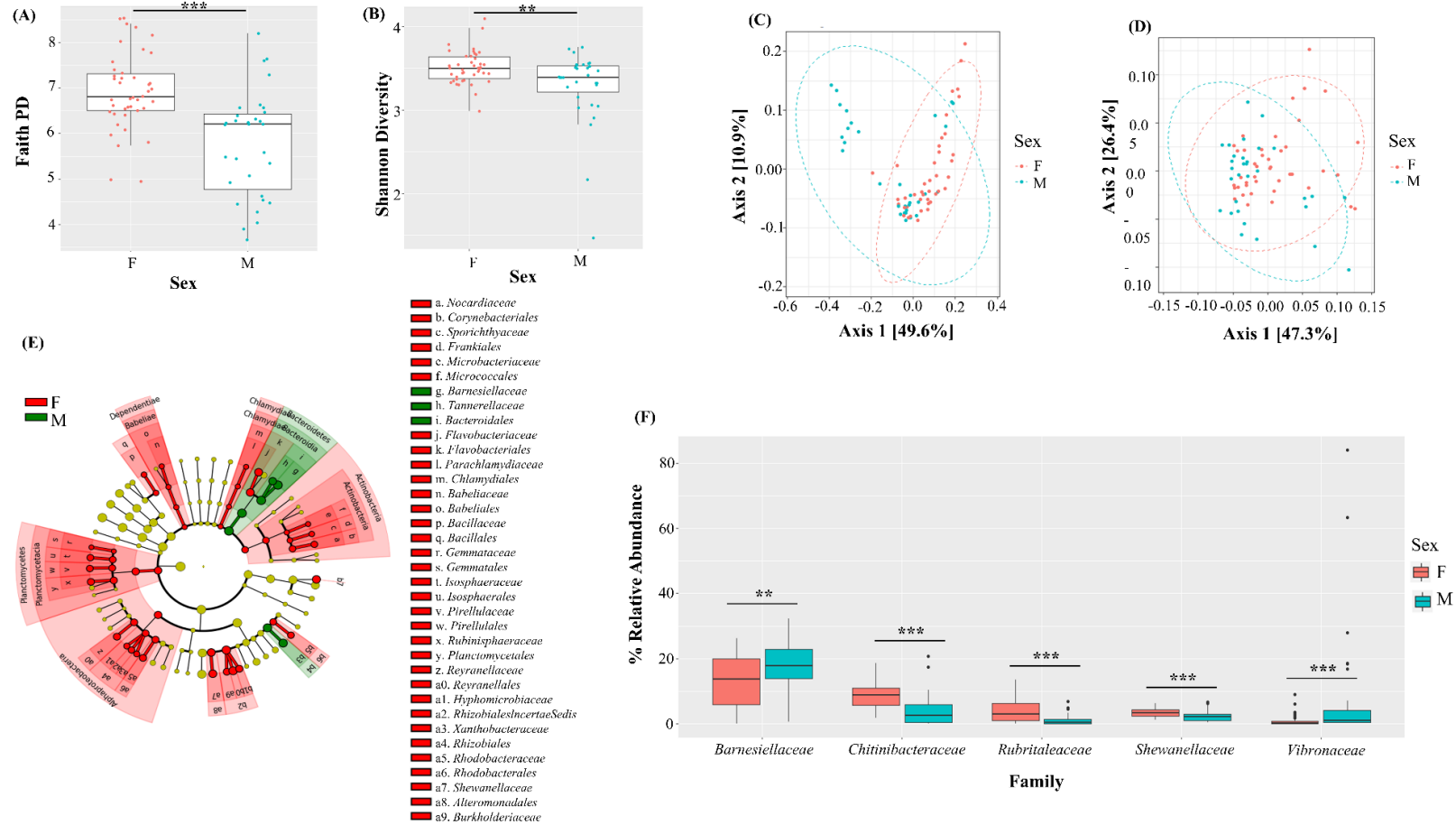
Group	Nominal exposure concentrations (µg/L)	Aqueous concentration 1 <sup>st</sup> sampling (mean ± SD, µg/L)	Aqueous concentrations 2 <sup>nd</sup> sampling (mean ± SD, µg/L)	Predicted plasma concentrations (µg/L)
High	100	90.1 ± 8.8 (n=29)	63.2 ± 9.2 (n=6)	1201.3
Middle	10	8.0 ± 0.9 (n=29)	4.2 ± 1.0 (n=6)	29.9
Low	0.01	0.006 ± 0.002 (n=8)	0.004 ± 0.0006 (n=3)	0.02
Control	0	0.002 ± 0.001 (n=8)	0.0008 ± 0.0002 (n=3)	0.006

### 3.4.2. *Host sex shaped gut microbiome*

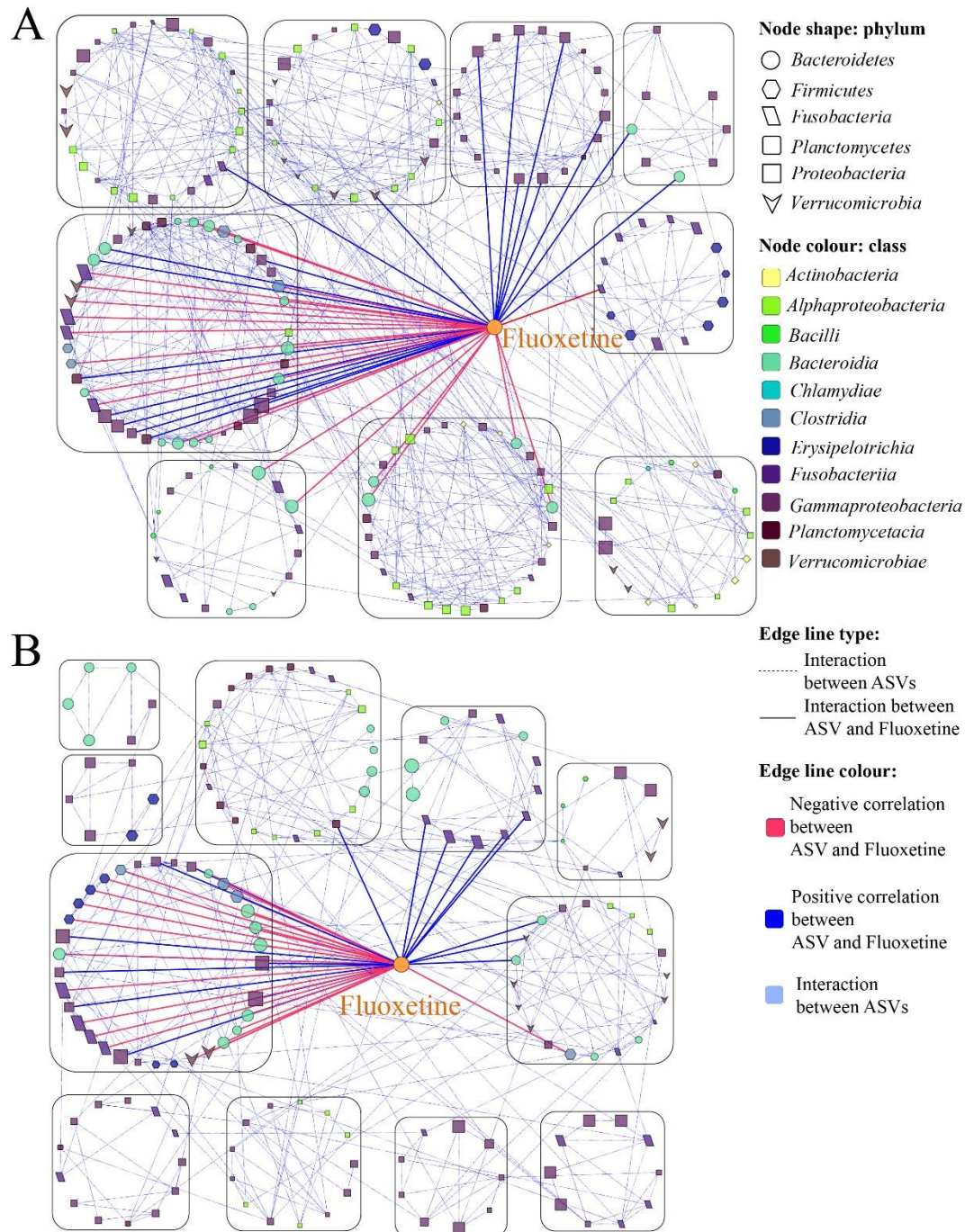
Female and male fish exhibited distinct compositions of gut microbial communities. In total, 386 ASVs were recovered for both female ( $n = 42$ ) and male fishes ( $n = 31$ ), with 5.2 million sequenced reads. 77.7% of reads survived after quality checking, filtering, merging and non-chimeric cleaning (Appendix F). Rarefaction at an even sequencing depth of 11,125 sequences per sample retained 377 ASVs (Appendix C). Alpha-diversities (Faith's Phylogenetic Diversity and Shannon diversity) of the gut microbiomes of females were greater than those of males (Wilcoxon one-tailed signed rank test,  $p < 0.01$ , Figure 3.1A and B). Beta-diversities (unweighted and weighted UniFrac distances) found significant separations between male and females (PERMANOVA, unweighted UniFrac distance,  $F = 11.96$ ,  $p = 0.001$ , Figure 3.1C; weighted UniFrac distance,  $F = 4.25$ ,  $p = 0.006$ , Figure 3.1D). Dispersion of unweighted UniFrac distances was heterogeneous while weighted values were homogenous between female and male fish (unweighted UniFrac distance; PERMDSIP test,  $F = 7.45$ ,  $P_{\text{perm}} = 0.008$ , weighted UniFrac distance; PERMDISP test,  $F = 0.03$ ,  $P_{\text{perm}} = 0.86$ ). Fifty-two bacterial families were differentially enriched in female fish compared to males, while three families were differentially enriched in

males compared to female fish (Figure 3.1E, LEfSe test,  $p < 0.05$  and  $\log_{10}$  transformed LDA score  $> 2$ ). Within the top 10 most abundant families, *Barnesiellaceae*, *Chitinibacteraceae*, *Rubritaleaceae*, *Shewanellaceae*, and *Vibrionaceae*, were significantly differentially abundant between male and female fish (Figure 3.1F). Female fish hosted a more complex microbial network than male fish where females had, 209 nodes, 562 edges and 5,378 neighbors and males had, 171 nodes, 339 edges and an average of 4,238 neighbors (Neighborhood Selection Network analysis, Figure 3.2A and Appendix L). The microbial Network of males contained fewer nodes per cluster corresponding to lower alpha diversity than compared to females.





**Figure 3.1** | Alpha diversity matrices comparing female (n = 42) and male (n = 31) fish: (A) Faith Phylogenetic Diversity;  $p = 6.127 \times 10^{-6}$  and (B) Shannon Diversity Index;  $p = 0.02$ . (C) Unweighted UniFrac and (D) Weighted UniFrac depicting the distances in microbial composition between female and male fish; Unweighted PERMANOVA;  $F = 11.96$ ,  $p = 0.001$ , Weighted PERMANOVA;  $F = 4.25$ ,  $p = 0.006$ . (E) Cladogram of taxonomic levels from a LEfSe analysis. Taxa in red are differentially and greater expressed in female fish and those in green are greater expressed in males. (F) Boxplot depicting relative abundant families found to be differentially expressed in females versus males. Significant correlations: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . F, female; M, male.



**Figure 3.2|** Neighborhood Selection Network analyses indicating an increase of fluoxetine to be significantly correlated to major clusters of class level taxa. Clusters from **(A)** female fish contain more nodes and thus higher alpha diversity. Clusters from **(B)** male fish hold less nodes representing less abundance of class level taxa. Red lines indicate a negative correlation between fluoxetine and abundance of classes while dark blue lines indicate a positive correlation. Light blue lines represent an interaction between nodes. Shapes of nodes indicate phylum level while color indicates the node's class.

### 3.4.3. Female gut microbiome response to fluoxetine exposure

Fluoxetine caused weak trends on select metrics of alpha diversity in female fish within the high exposure group. Faith PD showed greater abundance in the high (100µg/L) compared to the middle (10µg/L) exposure group,  $p < 0.05$ . No significant difference was observed between the control group and the high group, nor between the control group and the low and middle exposure groups when observing alpha diversity metrics (Figure 3.3A & B).

Exposure to aqueous fluoxetine significantly altered the relative percent composition of the gut microbiome of female fish (PERMANOVA test: unweighted distances,  $F = 4.39$ ,  $p = 0.001$ ; weighted distances:  $F = 5.38$ ,  $p = 0.001$ , Figure 3.3C & 3D). Beta diversity of the high exposure group was significantly different from that of all other groups (Table 3.2). No significant differential beta diversity matrices were found between control, low, and middle exposure groups within female fish. Five family level taxa were negatively associated with an increase in fluoxetine concentration while fifteen were positively correlated (Figure 3.3E). Several bacterial families were significantly different when comparing each exposure group to the control group (LEfSe test). *Akkermansiaceae*, *Peptostreptococcaceae* and *Barnesiellaceae* show a significant decrease in abundance in the high group while there was a significant increase in *Rubritaleaceae*, *Chitinibacteraceae*, *Shewanellaceae*, *Flavobacteriaceae*, and *Aeromonadaceae* in the high group compared to the control group. Abundance of *Akkermansiaceae* was also significantly less in the low group compared to the control group (Appendix G). Classes in the largest cluster of the female gut microbial network were significantly correlated with increasing concentrations of fluoxetine (Figure 3.2A), which is comparable with the heatmap of family level taxa (Figure 3.3E).

**Table 3.2**| Pairwise PERMANOVA and PERMDISP of Weighted and Unweighted UniFrac distances to determine significant differences of gut microorganisms between exposure groups within female or male fish. Bolded and starred p values indicate significance ( $p < 0.05$ ).

Host Sex	Group	Weighted UniFrac		Unweighted UniFrac	
		PERMANOVA	PERMDISP	PERMANOVA	PERMDISP
Female	Ctrl-Low	$F = 0.68$ , $p = 0.52$	$p = 0.76$	$F = 1.06$ , $p = 0.34$	$p = 0.28$
	Ctrl-Mid	$F = 0.76$ , $p = 0.56$	$p = 0.37$	$F = 1.13$ , $p = 0.27$	$p = \mathbf{0.006^*}$

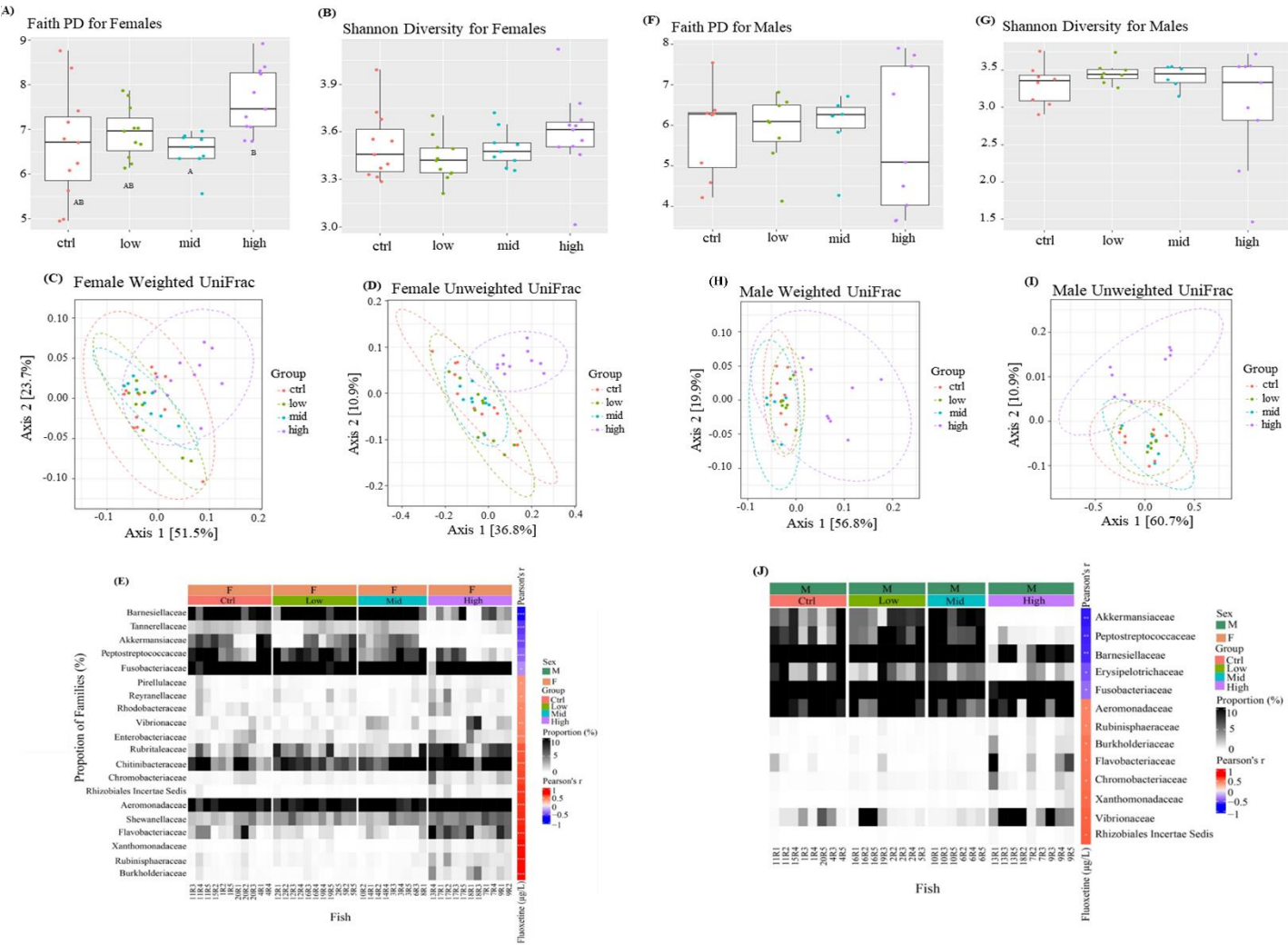
	<b>Ctrl-High</b>	<b>F = 8.16, <math>p = 0.002^*</math></b>	$p = 0.52$	<b>F = 6.36, <math>p = 0.001^*</math></b>	$p = 0.31$
	<b>Low-Mid</b>	F = 0.88, $p = 0.40$	$p = 0.57$	F = 1.7, $p = 0.10$	<b><math>p = 0.0008^*</math></b>
	<b>Low-High</b>	<b>F = 9.35, <math>p = 0.001^*</math></b>	$p = 0.32$	<b>F = 7.28, <math>p = 0.001^*</math></b>	$p = 0.80$
	<b>Mid-High</b>	<b>F = 9.56, <math>p = 0.001^*</math></b>	$p = 0.078$	<b>F = 9.43, <math>p = 0.001^*</math></b>	$p = 0.08$
<b>Male</b>	<b>Ctrl-Low</b>	F = 1.5, $p = 0.23$	$p = 0.99$	F = 0.92, $p = 0.396$	$p = 0.19$
	<b>Ctrl-Mid</b>	<b>F = 3.2, <math>p = 0.041^*</math></b>	$p = 0.8$	F = 0.65, $p = 0.53$	$p = 0.12$
	<b>Ctrl-High</b>	<b>F = 10.0, <math>p = 0.001^*</math></b>	<b><math>p = 0.01^*</math></b>	F = 1.55, $p = 0.183$	<b><math>p = 0.04^*</math></b>
	<b>Low-Mid</b>	<b>F = 2.6, <math>p = 0.049^*</math></b>	$p = 0.81$	F = 0.62, $p = 0.661$	$p = 0.52$
	<b>Low-High</b>	<b>F = 7.7, <math>p = 0.002^*</math></b>	<b><math>p = 0.01^*</math></b>	F = 1.98, $p = 0.136$	<b><math>p = 0.006^*</math></b>
	<b>Mid-High</b>	<b>F = 10.3, <math>p = 0.001^*</math></b>	$p = 0.05$	F = 1.7, $p = 0.187$	<b><math>p = 0.009^*</math></b>

#### 3.4.4. Male gut microbiome response to fluoxetine exposure

The gut microbiome composition of male fish was found to be altered by fluoxetine exposure. Alpha diversity (Faith PD and Shannon Diversity indices) of the microbial communities in guts of males were not significantly different among treatments (Kruskal-Wallis test,  $p > 0.05$ , Figure 3.3F & 3.3G). However, fluoxetine did significantly alter the matrix of weighted UniFrac distances among exposure groups (PERMANOVA,  $F = 7.14$ ,  $p = 0.001$ , Figure 3.3H). No significant differences were identified for Unweighted UniFrac distances for male fish (PERMANOVA,  $F = 1.41$ ,  $p = 0.218$ , Figure 3.3I). Pairwise PERMANOVAs indicate the significant differences in the weighted UniFrac were between the high exposure group and all other groups as well as between the middle exposure group and all other groups (Table 3.2). Homogeneity of multivariate dispersion was found significant for the weighted UniFrac between control and high as well as low and high exposure groups (PERMDISP,  $p = 0.01$ , Table 3.2).

Proportions of thirteen abundant families in the male gut microbiome were significantly correlated with aqueous concentrations of fluoxetine, five being negatively correlated and eight being positively correlated (Figure 3.3J). A LEfSe analysis found that within the top ten most relative abundant families, seven were differentially abundant when comparing each group separately to that of the control. *Akkermansiaceae*, *Erysipelotrichaceae* and *Peptostreptococcaceae* were more abundant in controls compared to the high exposure group while *Vibrionaceae* was more abundant in the high exposure group compared to the control (Appendix H). *Tannerellaceae* and *Akkermansiaceae* were more abundant in the middle group compared to the control and *Shewanellaceae* was more abundant in low exposure group

compared to control. The Neighborhood Selection Analysis revealed classes of taxa in the largest cluster in male fish were correlated with an increase in fluoxetine (Figure 3.2B).



**Figure 3.2** Alpha diversity matrices: Faith Phylogenetic Diversity for (A) females and (F) males, and Shannon Diversity Index for (B) females and (G) males. Beta diversity matrices: weighted UniFrac for (C) females and (H) males. Unweighted UniFrac distance matrices for (D) females and (L) males. Heatmaps (E, Females & J, males) depicting the correlation between proportion of families and the fluoxetine concentration fish were exposed to. Abundance of family level taxa are illustrated by the greyscale where black indicates higher abundance and white indicates lower abundance. Fish are sorted into their fluoxetine concentration groups which can be found vertically (ctrl, low, mid, high). A Pearson's correlation coefficient representing the association between fluoxetine exposure concentration and the proportion of families present can be found on the red-blue scale where red relates to a positive correlation and blue to a negative correlation between the two variables. Heatmaps for genus and ASV levels can be found in Appendices J and K. Significant level: \* < 0.05, \*\* < 0.01, \*\*\* < 0.001.

### 3.5. Discussion

The present study focused on whether fluoxetine can affect the gut microbiome of the fathead minnow in a sub-chronic aqueous exposure. Structures of male and female gut microbiomes were found to be significantly distinct while fluoxetine caused changes in community structure of both sexes when exposed to the highest concentration. Concentrations to which fish were exposed during this study that were close to the environmental quality standard values of interest (European Commission, 2000) for fluoxetine (0.01 µg/L) did not significantly alter the alpha-diversity or overall composition of gut microbiome. However, the high exposure concentration (100 µg/L), significantly altered alpha and beta diversity of the gut microbiome. The high exposure level was predicted to cause a plasma concentration of 1201.3 µg/L (Table 3.1; Predicted Plasma Concentrations), which is greater than the human therapeutic range (H<sub>T</sub>PC, 91-302 µg/L) (Margiotta-Casaluci et al., 2014; Pan et al., 2018)

Although the concentration of fluoxetine found to affect the gut microbiome in this study was substantially greater than concentrations observed in aquatic environments and greater than the H<sub>T</sub>PC, there is still relevance for understanding how this data can be used for cross-species extrapolation to predict similar effects on the evolutionarily conserved molecular targets in both



fish and mammals (Margiotta-Casaluci et al., 2014; Rand-Weaver et al., 2013). It also demonstrates a plausible mechanism of action for SSRIs to cause changes in the microbiome of vertebrates via potentially binding to the homologous transporter proteins expressed by select bacteria cells (Lyte et al., 2019; Lyte and Brown, 2018).

Abundant phyla identified in this study were consistent with other studies done on fathead minnow, including the two most prominent phyla *Fusobacteria* and *Proteobacteria* to be dominant within both males and females (Appendix I.A & B) (Bridges et al., 2018; DeBofsky et al., 2021; DeBofsky et al., 2020; Narrowe et al., 2015). These two phyla are also found to dominate in the zebrafish gut as well (Roeselers et al., 2011). *Bacteroidetes* and *Firmicutes*, the third and fourth most dominant phyla within fathead minnow in this study, are also prevalent in other fish gut microbiomes including rainbow trout, pinfish, silver perch, mummichog, and black sea bass (Colston and Jackson, 2016; Givens et al., 2015). Tryptophan metabolism pathways have been found to be enriched in these four dominant phyla in the fish gut (Kaur et al., 2019), which suggests that the fish gut microbiome can produce neuroactive compounds influencing the gut-brain axis (Bastiaanssen et al., 2020).

When comparing the differences between sexes in the control exposure groups only, as well as comparing males and females within each dosage group, significant differences were identified observing diversity matrices. This could be due to fluoxetine differentially impacting male and female gut microbiomes; thus, it is necessary to separate the sexes to better assess how fluoxetine influences the gut microbiome. In other studies sex has also been identified to play a major role in composition of the gut microbiome (Bolnick et al., 2014; DeBofsky et al., 2020; Gao et al., 2018; Jašarević et al., 2016; Lozano et al., 2018) further demonstrating the importance of separating males and females when analyzing microbiome data where, lower alpha diversity in males has been reported in fish, mice, and humans (de la Cuesta-Zuluaga et al., 2019; DeBofsky et al., 2020; Li et al., 2016; Yurkovetskiy et al., 2013). It has been postulated that hormonal changes during puberty can determine the structure of the gut microbiome potentially through androgen receptor activity (de la Cuesta-Zuluaga et al., 2019; Markle et al., 2013; Yurkovetskiy et al., 2013). Within families enriched in the female fish gut microbiome in this study, *Burkholderiaceae* and *Bacillaceae* are predicted to have a substantial capability in metabolizing tryptophan within the vertebrate gut, as well as *Pseudomonadaceae* (Kaur et al.,

2019), found in both sexes. This finding indicates that some bacteria, potentially more predominant in female fish, may be more affected by tryptophan and serotonin levels within the body.

In general, there may be two major direct routes of interaction between fluoxetine and gut microbiota, including, antimicrobial potential of fluoxetine and the interaction with metabolite pathways generating neuroactive compounds (Ait Chait et al., 2020; Zhang et al., 2021). Results of this study suggest antimicrobial action of fluoxetine may have caused significant alteration of gut microbiomes of both females and males, which resulted in changes in structures of the microbial community in guts of fish exposed to the highest concentration. Furthermore, negative relationships between concentrations of fluoxetine and proportion of taxa (ASV) were observed in both female and males (Figure 3.2A & B, Figure 3.3E & J). Relative proportions of *Akkermansiaceae* and *Peptostreptococcaceae* observed here were negatively correlated with aqueous concentrations of fluoxetine, consistent with the antimicrobial activity of antidepressants found in previous studies (Ait Chait et al., 2020; Zhang et al., 2021). It is hypothesized that the proposed antimicrobial capabilities of SSRIs is a result of binding to homologous serotonin reuptake transporters on bacterial cells. *In vitro* colonization of certain bacteria, such as *Lactobacilli* and *E. coli*, were found to be inhibited when exposed to fluoxetine due to its ability to block a biogenic amine transporter found on some bacterial cells, homologous to that of the serotonin reuptake transporter (SERT) in vertebrates (Cussotto et al., 2018; Lyte and Brown, 2018).

The representative bacterial species in human gut microbiomes are sensitive to non-antibiotic drugs, such as antidepressants (Maier et al., 2018), which suggests that alterations of gut microbiota by fluoxetine might influence fitness of the host. The species *Turcibacter sanguinis*, for instance, within the family *Erysipelotrichaceae*, found to be negatively associated with fluoxetine in males in this study, has recently been identified to play an important role in serotonin signaling, holds ortholog transporters to SERT and has previously been found to be inhibited by SSRIs (Fung et al., 2019; Jones et al., 2020). Changes in the presence of *Akkermansia muciniphila*, in the family *Akkermansiaceae* found to also be negatively affected by fluoxetine in this study, can adversely affect host health. An increase in *A. muciniphila* is linked to fatty acid oxidation while a decrease could be associated with inflammatory markers,



metabolic alterations, and potential disease progression (Schneeberger et al., 2015; Sivixay et al., 2021). Importantly, *Akkermansiaceae* can regulate host serotonin through an outer membrane protein, Amuc\_1100 (Wang et al., 2021).

Due to the distinct composition of gut microbiome between female and male fish, response to fluoxetine exposure presented sex-specific patterns at each taxa-level. *Clostridiales* (Phylum *Firmicutes*), found here to be significantly less in females exposed to the greatest concentration of fluoxetine, compared to controls, has been found to respond to host serotonin fluxes suggesting it might have transporters similar to that of SERT (Fung et al., 2019). Abundances of Proteobacterial families: *Shewanellaceae* and *Aeromonadaceae* in females and *Vibrionaceae* in males were positively correlated with concentrations of fluoxetine and have been found to be associated with anxiety, inflammation, and pathogenesis, respectively (Colwell and Grimes, 1984; De Palma et al., 2017; Reid et al., 2008; Song et al., 2016). When exposed to the greatest concentration of fluoxetine, there were several more families altered in abundance in females compared to males (Figures 3.3E & J, Appendices G & H). The greater effect of fluoxetine on females could be a result of sex-hormones playing a role in the microbial makeup. Due to a greater alpha diversity in females, there is a larger potential for fluoxetine to influence these bacterial communities. It has been identified that composition of microbial composition of guts of males and females might be affected differently by pharmaceuticals (Sinha et al., 2019).

Results of previous studies have revealed that fluoxetine can cause anxiolytic behavior, appetite suppression, reproduction impairments and modulation of the predator-prey response in fish (Lister et al., 2009; McDonald, 2017; Mennigen et al., 2010; Pelli and Connaughton, 2015; Weinberger and Klaper, 2014). However, similar to this study, some of the adverse effects identified in those exposures occurred at concentrations greater than those typically observed in the environment and exposed for relatively short durations of less than 35 days. Due to continuous release of pharmaceuticals into aquatic environments and their ability to bioconcentrate (Pan et al., 2018), chronic and multigenerational studies at environmentally relevant concentrations would be of value (Polverino et al., 2021; Silva et al., 2015; Tan et al., 2020). A future chronic study should be conducted at environmentally relevant concentrations to reveal whether the presence of fluoxetine may perturb the gut microbiome of fish long-term. Work should also be done addressing tissue and blood concentrations, which would also help interpret

effects of fluoxetine across vertebrate species that hold conserved molecular pharmaceutical targets (Rand-Weaver et al., 2013). Finally, functional capabilities of gut microbiota perturbed by fluoxetine through integrated multi-omics techniques would be valuable to further understand the connection between the microbiome and its host (Whon et al., 2021). For example, if we are able to understand which microbes are inhibited in colonization and growth, we can further identify what function these biota hold in the body using transcriptomics, proteomics as well as metabolomics techniques. These approaches allow us to identify the transcription of DNA to RNA (transcriptomics), the translation of RNA to proteins, essential for cell and tissue growth (proteomics), and the metabolites that form after cellular processes (metabolomics). These techniques are valuable in identifying the function of the microbial communities making up the microbiome and allow us to further appreciate and utilize the microbiome in the context of risk assessment.

### **3.6. Conclusions**

Shifts in dominant taxa in the gut microbiomes of fathead minnow (*P. promelas*) were observed in individuals exposed to the greatest concentration of fluoxetine (100 µg/L). The lowest (0.01 µg/L), which was more representative of environmentally relevant concentrations, and middle concentration (10 µg/L) did not significantly alter the gut microbiomes of fathead minnows. This study demonstrated that fluoxetine can affect the gut microbiome, yet at concentrations greater than observed in the environment. Due to evolutionarily conserved transporter proteins in vertebrates and microbiota, it can be predicted that certain gut microbiota in other vertebrates may be impacted by fluoxetine as well. Future long-term studies will help determine if fluoxetine, at environmental relevant concentrations, can affect the gut microbiome of fish. A reduction in several commensal bacteria and an increase in pathogenic and inflammatory related taxa indicates that host health may be perturbed long term. The gut microbiome plays a crucial role in host immune and nervous systems; thus, it is pertinent to establish an understanding of the types of microbiota colonizing the gut and their functional capabilities to better understand how host homeostasis is affected through xenobiotic exposure.

## CHAPTER 4: GENERAL DISCUSSION

#### **4.1. Project focus and objectives**

With the knowledge of a microbiome-gut-brain-axis, where a bi-directional influential relationship takes place between the brain and the microbial communities living within the gut, it is critical to better understand how these microbes are influenced when their host is exposed to toxicants. Only in recent years has the importance of the gut microbiome become understood in the field of ecotoxicology (Evariste et al., 2019). To our knowledge this is the first study conducted to determine the impact of antidepressants, found downstream of wastewater treatment plants, on the gut microbiome of a commonly studied fish, the fathead minnow (*Pimephales promelas*). The focus of this research was to heighten an understanding of the effects on the gut microbiome when its host is exposed to a xenobiotic and to give stronger evidence of the importance of microbiome studies in the field of ecotoxicology. The main objectives of this study were to better understand if (1) if the antidepressant, fluoxetine, can impede growth of bacterial cells, cultured from the gut of fathead minnows, *in vitro*, and, using 16S rRNA metabarcoding techniques, (2) determine if differences exist between the gut microbiome of female and male fathead minnow, (3) identify if fluoxetine can cause dysbiosis of the gut microbiome when fish are exposed to three different concentrations of fluoxetine, and (4) compare sex-specific effects of fluoxetine on the gut microbiome.

#### **4.2. Summary of findings**

##### **4.2.1. Minimum inhibitory concentrations**

The basis of running a minimum inhibitory concentration assay was established due to the understanding that some bacterial cells, including commensal bacteria living within the gut microbiome, express homologous proteins to those of the neurotransmitter:sodium symporters, such as the selective serotonin reuptake transporter found on numerous types of cells in vertebrates (Lyte and Brown, 2018; Zhou et al., 2009). Previous studies have been conducted, specifically looking at mammalian commensal microbiota where SSRIs can bind to these transporters on bacterial cells, impeding their growth (Bohnert et al., 2011; Cussotto et al., 2018; Lyte and Brown, 2018; Munoz-Bellido et al., 2000). Bacteria previously cultured from the gut microbiome of fathead minnow were exposed to a dilution series of fluoxetine ranging from 0.015 to 128 µg/mL. After overnight inoculation, cell growth was determined, concluding that growth of all cultured bacteria was indeed inhibited at concentrations of 32 µg/mL and higher of

fluoxetine. It was previously hypothesized that the potential reasons for inhibition of growth are due to the efflux pump inhibition by these SSRIs and the potential that these bacterial cells contain the sodium symporters homologous to SERT which SSRIs can then bind to (Lyte and Brown, 2018; Munoz-Bellido et al., 2000). The results from this preliminary study established a basis to better understand if such mechanisms of growth inhibition can occur *in vivo* when the host organism for these microbes is exposed to fluoxetine. Thus, a second study was conducted exposing fathead minnow to select concentrations of fluoxetine.

#### ***4.2.2. Analyzing sex differences and the effects of fluoxetine on the gut microbiome of fathead minnow***

Fluoxetine was found to have a significant effect on the gut microbiome of fathead minnows at the highest exposure concentration (nominal concentration of 100 µg/L) where the microbial makeup was found to be significantly shifted away from the other groups. After a baseline understanding that fluoxetine can inhibit bacterial growth *in vitro*, a fish study was conducted exposing fathead minnows to three different concentrations of fluoxetine as well as a control. After 28 days, fish were euthanized, GI tracts were extracted, DNA extraction, amplification, purification and sequencing was run, and bioinformatics was then performed to identify if fluoxetine can influence the microbial community structure of the gut microbiome of fathead minnows. It was determined that not only did fluoxetine induce changes in the microbiome at the highest concentration, but the gut microbiome of female and male fish were found to be distinct. The female gut microbiome was found to have greater alpha diversity, and the types of microbiota making up these communities were found to be separating away from each other when observing beta-diversity. These findings coincide with other studies, demonstrating that the makeup and abundance of bacteria are significantly different when comparing male and female hosts (Bolnick et al., 2014; DeBofsky et al., 2020; Gao et al., 2018; Jašarević et al., 2016; Lozano et al., 2018). The differences found between sexes may be due to hormonal changes where the gut microbiome has been found to be influenced by androgen receptor activity (de la Cuesta-Zuluaga et al., 2019; Markle et al., 2013; Yurkovetskiy et al., 2013). Due to differences in microbial makeup of the gut microbiome of male and female fish, it was critical to separate the two sexes when identifying if fluoxetine impacted these microbial communities. Alpha diversity was not seen to be considerably impacted by fluoxetine exposure at any concentration

for either sex. Interestingly, the variability in alpha diversity within male fathead minnows was quite prevalent in the highest exposure group, where fluoxetine may have caused inconsistencies in the abundance of bacteria present. When observing beta diversity, we found that the communities of bacteria present were remarkably changed by the highest concentration of fluoxetine for both males and females. Several bacterial families were found to be significantly altered in their presence at this high concentration, for instance, *Akkermansiaceae* and *Peptostreptococcaceae* are found to be significantly less abundant in the highest exposure group compared to controls in both sexes. In females, proportions of microbiota influenced by fluoxetine were greater than in males.

Using a network analysis to identify how fluoxetine is impacting clusters of correlated class level taxa, fluoxetine was found to majorly influence and be associated with dominant classes in the gut microbiome of both sexes. This was a major finding for many of these dominant classes hold influential roles on other less predominant clusters of families. Their influence could potentiate a cascade effect where, eventually, fluoxetine may subsequently impact other bacterial classes.

As previously hypothesized, fluoxetine may be able to impede growth and thus impact colonization of certain bacteria through its ability to bind to sodium symporters homologous to that of SERT found on some bacterial cells (Lyte et al., 2019; Lyte and Brown, 2018). Previous studies found that certain bacteria respond to serotonin fluxes of its host, suggesting that they may hold similar transporters to that of SERT (Fung et al., 2019; Wang et al., 2021). These taxa are included in the families *Clostridiaceae* and *Akkermansiaceae* which were also identified to be diminished in this study. Interestingly, several families in the phyla *Proteobacteria* that are associated with inflammation, pathogenesis, and anxiety (Colwell and Grimes, 1984; De Palma et al., 2017; Reid et al., 2008; Song et al., 2016) were found to be more abundant in the highest exposure group; while, *Akkermansiaceae*, a bacterial family found to be almost completely eradicated in the highest exposure group for both sexes, is critical for fatty acid oxidation and a decrease is correlated with inflammatory markers, disease, and metabolic alterations (Schneeberger et al., 2015; Sivixay et al., 2021).

Although the dysbiosis of the gut microbiome related to fluoxetine exposure was only found at the highest exposure concentration which is not environmentally relevant, this work can still

be used as a proof-of-concept study where we can take away the fact that fluoxetine can indeed impact the gut microbiome. Importantly, this work can be used as a read-across hypothesis, where we can extrapolate these findings to other vertebrate studies predicting outcomes on evolutionarily conserved molecular and bacterial targets (Margiotta-Casaluci et al., 2014). Also, continuous release of SSRIs, and the reality of pharmaceutical mixtures within the environment, could allow for an increase in the availability and uptake these compounds, causing higher levels of SSRIs in the body (Backhaus, 2014; Daughton, 2002; Ding et al., 2017; Paterson and Metcalfe, 2008; Schultz et al., 2011; Simmons et al., 2017).

#### **4.2.3. Linking MIC and metabarcoding results**

Antimicrobial effects were found in both the minimum inhibitory concentration assays and the microbiome analysis conducted. However, the results of both studies hold some incongruencies. For example, at a genus level, *Aeromonas* and *Plesiomonas* were found to increase in abundance with increasing concentrations of fluoxetine in the metabarcoding study (Appendix K) while the species, *Aeromonas veronii*, *Aeromonas ichthiosmia*, and *Plesiomonas shigellodes* were found to be inhibited by fluoxetine in the MIC tests. These results, however, are not entirely comparable as the concentrations used held a difference of approximately a thousand-fold. Lyte et al., (2019) also found similar inconsistencies when comparing *in vitro* antimicrobial studies to a 16S metabarcoding analysis where *Escherichia* was found to be inhibited at extremely high concentrations *in vitro* yet not at the human therapeutic dose when mice were exposed. MIC testing is used to understand bacteriostatic effects, where a compound can prevent visual growth of bacteria, requiring a high concentration of the compound of interest. It is possible that these bacteria may be influenced differently at a substantially higher concentration where a biphasic or hormesis effect may occur.

Strain specific effects could also be happening that cannot be deciphered at the genus level. Although the genera *Aeromonas* and *Plesiomonas* are higher in abundance at increasing concentrations of fluoxetine in the metabarcoding study, using metataxonomic techniques, we cannot verify what species we are observing. Intriguingly, in a previous study, two different species of the genus *Lactobacillus* were found to differentially express biogenic amine transporters (Lyte and Brown, 2018). Thus, although a greater abundance of genus level *Aeromonas* and *Plesiomonas* were found in our metabarcoding study, a different story may be

unfolding at a species level which could be why we see inhibition of growth of certain species in these genera in the MIC assays. The two studies conclude that fluoxetine can impede growth of certain bacteria found in the gut of fathead minnows, however, continued research needs to be conducted exploring the mechanism behind this impediment.

#### **4.3.Limitations of research and recommendations for future work**

##### ***4.3.1. Development limitations to identify transporters on bacterial cells***

Although our hypothesis that growth inhibition of bacterial cells when exposed to fluoxetine is caused by transporter inhibition, we were not able to verify the true source of this impairment. Lyte and Brown (2018), were able to identify monoamine transporters on a *Lactobacillus* strain using fluorescence-based assays with transporter-specific fluorophores. One of the objectives after running the MIC testing of fluoxetine was to use similar methods to see if the bacterial cells from this study held these transporters as well. Unfortunately, to run this assay, the bacteria must be able to make biofilms or at least adhere to the bottom of a well plate. Due to trouble maintaining adhesion of bacterial cells to 24 and 96 well plates, this assay was not able to be performed. Future work should be conducted to identify these transporters, potentially using agarose gel allowing for adhesion and growth of bacterial cells before running the fluorescence-based assay. Running gene-specific amplification using quantitative PCR (qPCR) techniques with primers designed for the monoamine transporters of interest could be another valuable option. However, to run this analysis, genes encoding for transporters of interest must be identified (Lyte et al., 2019; Peirson and Butler, 2007). Another possibility would be to use transcriptomic or proteomic techniques which can determine gene and protein expression from bacterial species (Kolker et al., 2006; Mishra et al., 2019).

##### ***4.3.2. The pros and cons of 16S rRNA metabarcoding***

Advances in high-throughput sequencing has allowed for an upsurge of studies regarding the microbiome across scientific fields (Ranjan et al., 2015). There are currently two predominant ways to analyze microbiome data, the first being 16S rRNA amplicon sequencing which relies on a marker gene, the 16S rRNA gene, present in almost all bacteria (Janda and Abbott, 2007; Kuczynski et al., 2011). The second method is whole genome shotgun sequencing where the entire metagenome is sequenced (Kuczynski et al., 2011). Both techniques have their



advantageous and limitations. The 16S rRNA technique, used for this study, is currently the most common taxonomic identification tool (Brumfield et al., 2020). Amplicon sequencing continues to be the more inexpensive technique (Brumfield et al., 2020; Van Nimwegen et al., 2016) and may be the better option when running studies with limited sequencing reads (Durazzi et al., 2021). Using universal primers, we can amplify certain hypervariable regions of the 16S rRNA gene and through bioinformatic processing, we can align the obtained sequences against sequence databases (Brumfield et al., 2020; Kuczynski et al., 2011) including SILVA Ribosomal RNA and Gene Database Project (Quast et al., 2013) or Greengenes (DeSantis et al., 2006). Although this tool may be the more cost effective and the practical choice, whole genome sequencing tends to be a more accurate and predictable method.

Whole genome shotgun sequencing uses all DNA which is then fragmented, sequenced and aligned independently (Brumfield et al., 2020). Shotgun sequencing, although more expensive, tends to be the more advantageous sequencing technique, as long as a sufficient amount of reads are obtained (Durazzi et al., 2021). Comparison studies have found that shotgun sequencing yields more accuracy in taxonomic classification at lower levels including genus and species detection, can better identify diversity of taxa found, and can predict protein features, genes and functions of the microbial communities (Brumfield et al., 2020; Durazzi et al., 2021; Kuczynski et al., 2011; Ranjan et al., 2015). Additionally, unlike 16S sequencing, shotgun techniques can also identify viruses, protozoa and fungi (Ranjan et al., 2015). Although metagenomic sequencing data can be assessed to predict function of microbiota, due to individual variation of these microbes where highly related bacteria can hold significantly different functions, it is not adequate to rely on tools that use community composition to predict function (Duperron et al., 2020). Instead, a multi-omic approach may be better suited where we can identify gene and protein expression as well as metabolites microbiota produce to better explain the functional capabilities they may hold.

#### ***4.3.3. Future work incorporating omics techniques for functional analyses***

To assess functional profiles of the gut microbial communities, future work should be conducted using other omic techniques including metatranscriptomics, metabolomics, metaproteomics. A multi-omics approach is challenging, yet the ability to use these techniques could give a more accurate and reliable profile of the function of these microbes when their host

is exposed to a toxicant (Duperron et al., 2020). A multi-omic approach allows us to not only observe the abundance of genes, but we can also evaluate gene expression, expression of proteins, and metabolite production (Malmuthuge and Guan, 2016). For instance, metatranscriptomics, an RNA-based method, identifies microbiota activity through transcription and expression of genes (Malmuthuge and Guan, 2016; Whon et al., 2021). Metagenomics may be able to identify abundance of genes, however, several of these genes may not be actively expressed, which is where metatranscriptomics comes into play (Franzosa et al., 2014; Malmuthuge and Guan, 2016).

Through the use of metaproteomics, studying the expression of proteins produced by the microbiome, helps assess the direct activity of these microbes in the gut (Whon et al., 2021). Proteins identified using metaproteomic techniques hold highly influential roles within their host, for example, inflammatory response proteins and mucosal integrity proteins expressed by microbes can influence chronic inflammation in the host organism (Erickson et al., 2012; Lai et al., 2019). Use of shotgun metaproteomics has also identified several proteins expressed by microbes correlated with energy production, immune responses, and carbohydrate metabolism (Lai et al., 2019; Verberkmoes et al., 2009).

Metabolomics is the study of metabolites; molecules transformed during metabolism processes (Whon et al., 2021). Most metabolomic studies are currently conducted using nuclear magnetic resonance (NMR) spectroscopy and LC-MS techniques. Using metabolomic methods in gut microbiome studies hold some trepidations; to date it is difficult to differentiate which microorganism a metabolite came from due to the complex mixture of the gut contents of the host and bacterial secretions (Whon et al., 2021; Yan et al., 2016). Identification of metabolites can also be tricky due to how quickly they can degrade during the process of extraction (Yan et al., 2016). However, because metabolites are, in essence, what is left behind after a cellular response, metabolomic methods hold value in microbiome studies, for it can give a better insight into the metabolic processes that may be influenced by toxicants (Peters et al., 2019).

#### **4.4. Concluding statements**

Pharmaceuticals, such as SSRIs, are increasingly and continuously being released into aquatic environments. With that, there have been a plethora of environmental toxicology studies conducted on how such pharmaceuticals can impact numerous endpoints including behavior,

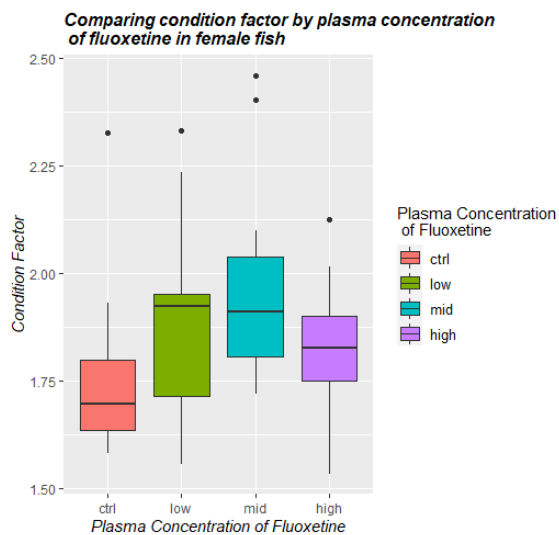
development, and reproduction. Although it is known that the gut holds the majority of serotonin synthesis and several bacterial species hold homologous transporters to that of the serotonin reuptake transporter that SSRIs bind to, no ecotoxicological study has previously been conducted to determine how the fish gut microbiome is influenced when the host is exposed to an SSRI. In fact, the incorporation of the gut microbiome has only recently been recognized in the field of ecotoxicology. The gut microbiome is a critical element within host organisms and can interact with the immune system, nervous systems, GI function, and brain. Because of our awareness of the interconnectedness of the gut microbiome and homeostasis of its host, it is vital to better understand how toxicants may impair the gut microbiota and how such perturbations can then influence the host organism. This study demonstrated that the xenobiotic, fluoxetine, can impede bacteria cell growth *in vitro* and cause dysbiosis of the gut microbiome. However, it is important to note, that the concentration of fluoxetine at which the gut microbiome was affected is far greater than anything seen in the environment. This stated, due to continuous release of fluoxetine into the environment and its ability to bioconcentrate, a long-term exposure may give us a better understanding of how this SSRI may affect the gut microbiome of aquatic organisms. Lastly, this work can be applied as a cross-species extrapolation study, for all vertebrates hold serotonin systems and microbiomes allowing us to extrapolate from such evidence found here and apply it to other organisms. This work gives us a first look regarding how fluoxetine, a common SSRI found within waterbodies, may impact the gut microbiome of fish.

## APPENDICES

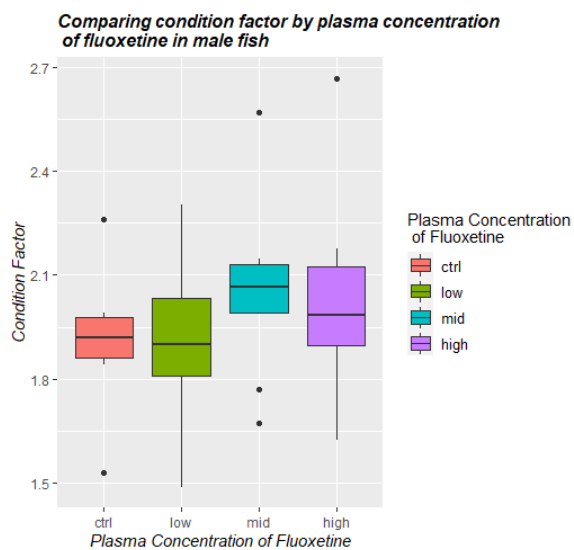
**Appendix A**| Detailed parameters for LCMS quantification of fluoxetine for both chapter 2 and 3:

Samples were ionized by positive mode heated electrospray ionization (HESI) with the following source parameters: sheath gas flow = 20; aux gas flow = 5; sweep gas flow = 1; aux gas heater = 300°C; spray voltage = 3.5 kV; S-lens RF = 60; and capillary temperature = 350°C. A targeted-SIM and PRM (collision energy = 20) method at 60,000 resolution, AGC target =  $1 \times 10^6$ , and max injection time = 100ms was used to monitor  $[M+H]^+$  precursor and product ions of fluoxetine ( $m/z$  310.141  $\rightarrow$  148.112) and fluoxetine-d5 ( $m/z$  315.173  $\rightarrow$  153.143). Precursor and product ions were used for quantification and confirmation, respectively.

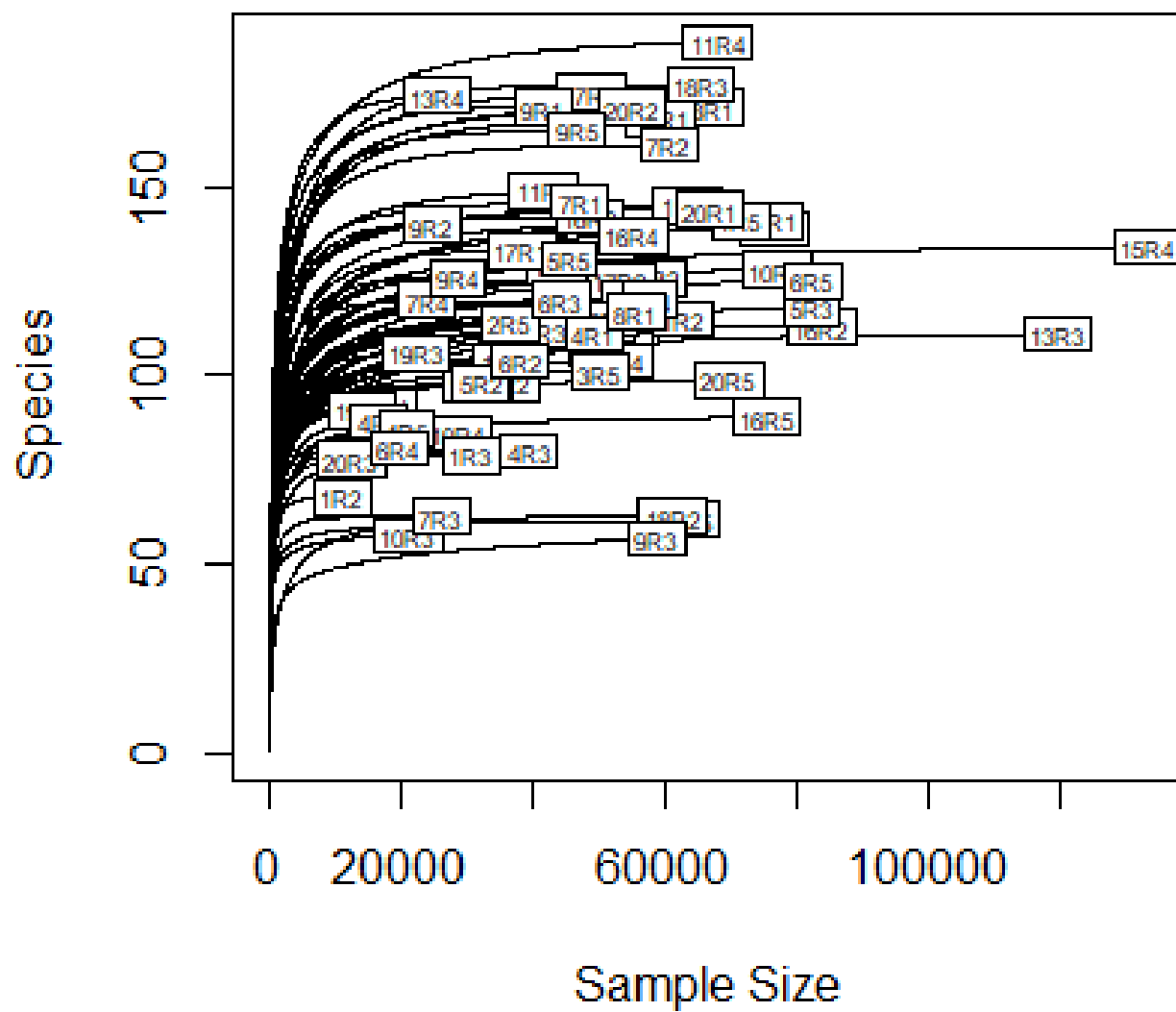
A



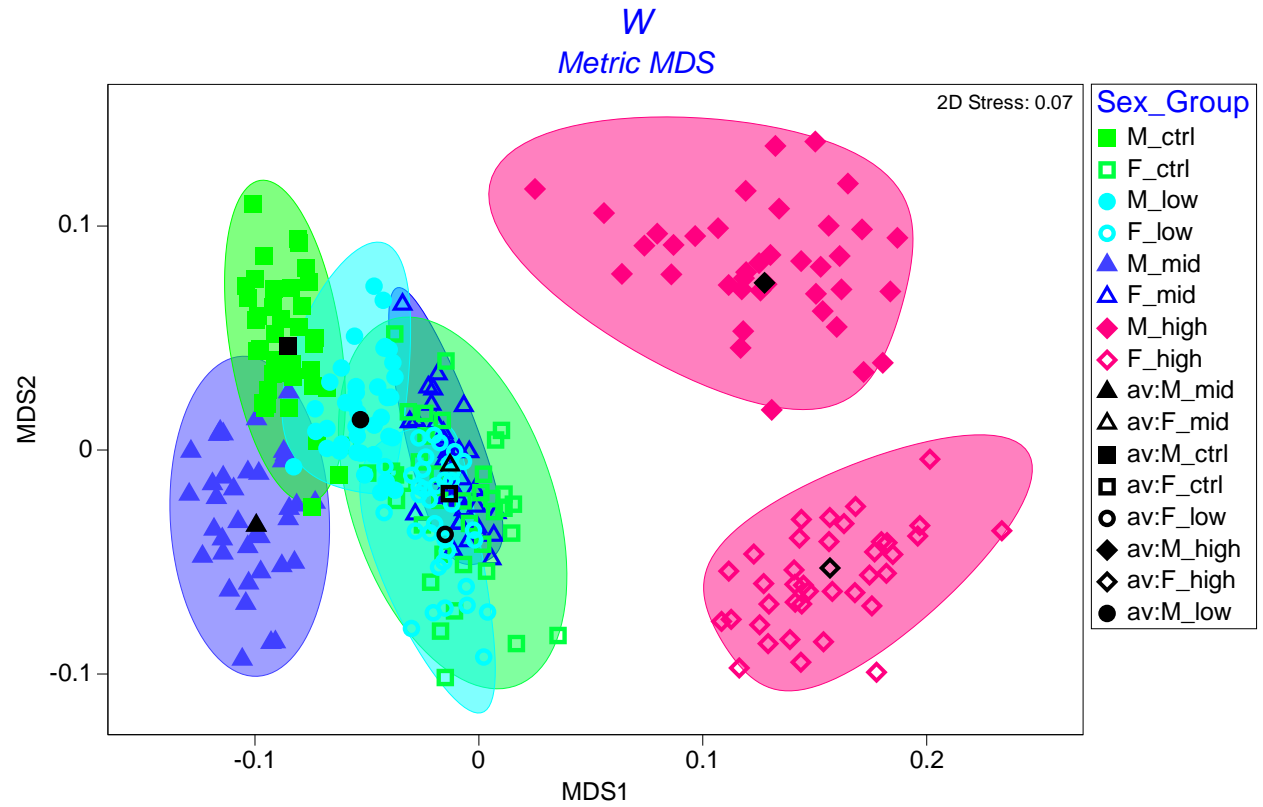
B



**Appendix B** | Boxplots comparing condition factor against exposure concentrations (A) female fish (ANOVA test,  $p > 0.05$ ;  $n=45$ ); (B) male fish (ANOVA test,  $p < 0.05$ ;  $n=32$ ). No significant differences were identified between concentration of fluoxetine and condition factor of male and female fish. (Chapter 3).

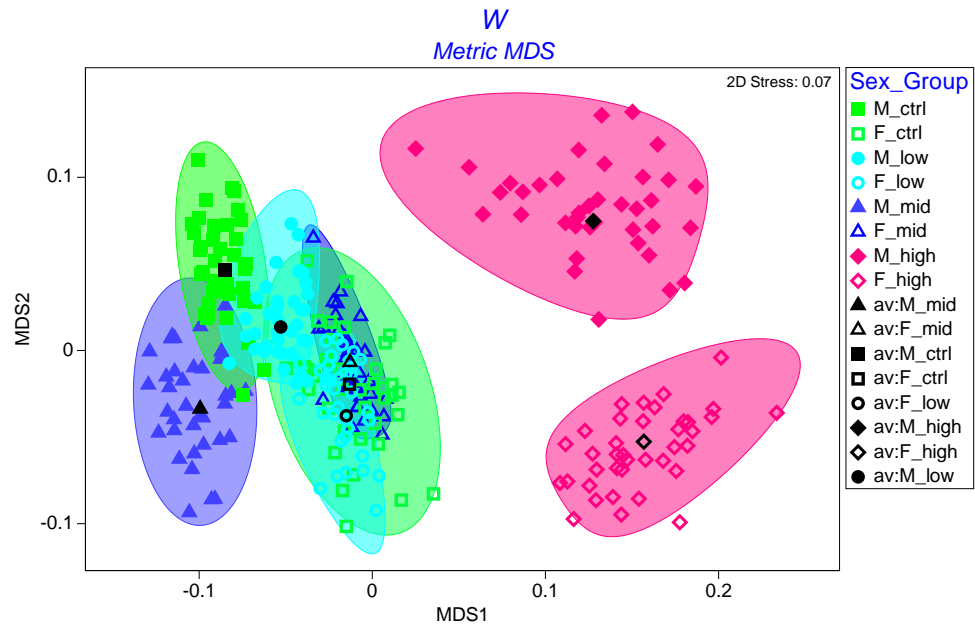


**Appendix C** Rarefaction curve for all samples in the fathead minnow microbiome study (chapter 3).



**Appendix D**| NMDS plot based on bootstrap averaged beta-diversities to show the variance of each group (chapter 3).





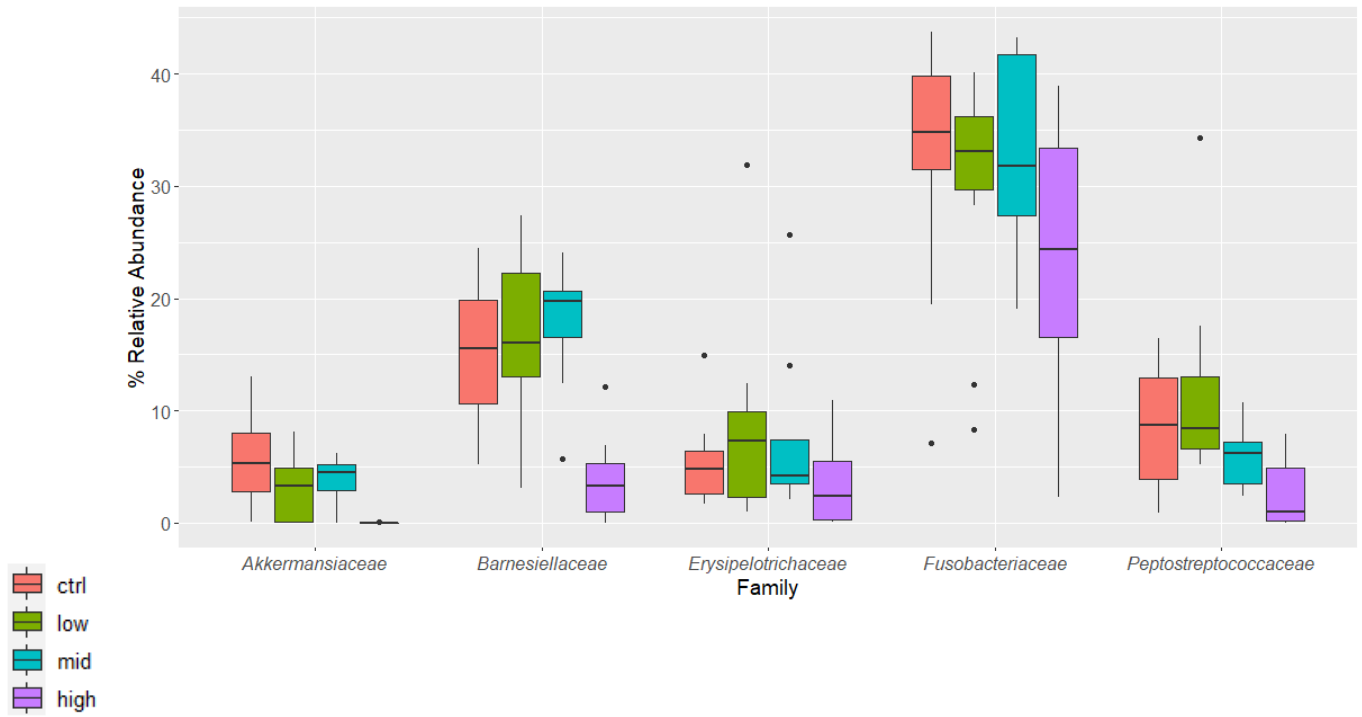
**Appendix E|** NMDS plot based on bootstrap averaged beta-diversity. Males in the greatest exposed group hold larger variance than females in the greatest exposed group (chapter 3).

**Appendix F** | Table showing reads per sample before and after filtering, denoising, merging and removing of chimeras. (Chapter 3)

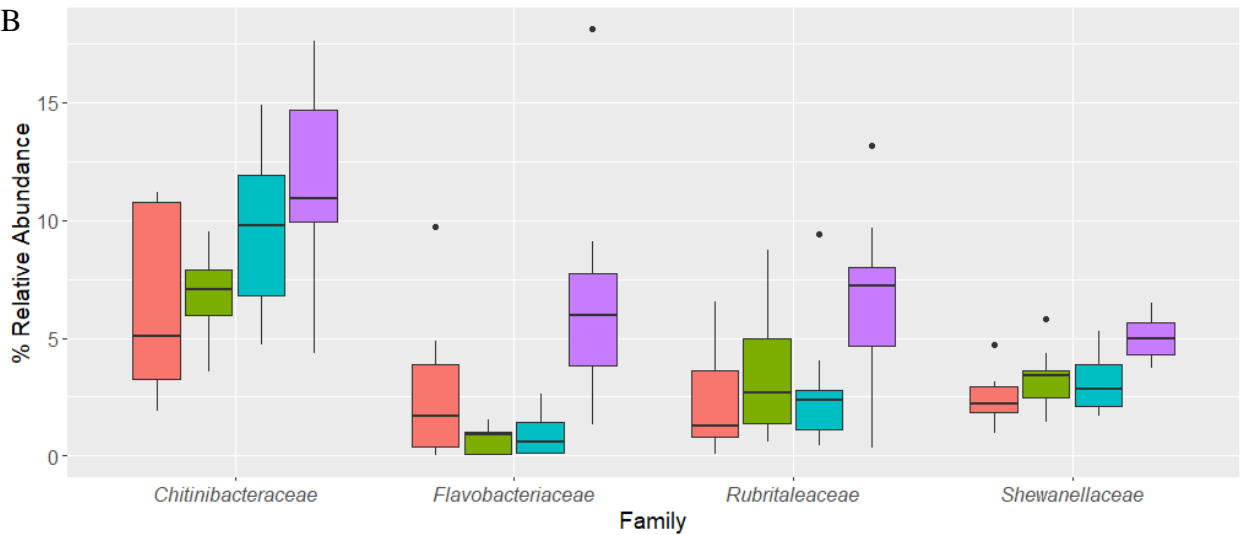
sample-id	input	filtered	percentage of input passed filter	denoised	merged	percentage of input merged	non-chimeric	percentage of input non-chimeric
10R1	23771	20814	87.56	20530	19716	82.94	18758	78.91
10R2	108046	94679	87.63	93925	92106	85.25	83333	77.13
10R3	27899	24375	87.37	24045	23342	83.67	22411	80.33
10R5	76565	66718	87.14	66104	64527	84.28	59375	77.55
11R1	54800	48121	87.81	47650	46386	84.65	44548	81.29
11R2	84409	74539	88.31	74044	72392	85.76	66451	78.73
11R3	64056	56303	87.9	55652	53740	83.9	47062	73.47
11R4	85331	73265	85.86	72779	72093	84.49	71669	83.99
11R5	82650	72023	87.14	71624	70494	85.29	68829	83.28
12R1	91713	79911	87.13	79587	79090	86.24	78461	85.55
12R2	72499	64053	88.35	63379	61684	85.08	54701	75.45
12R3	60670	53625	88.39	53380	53040	87.42	52710	86.88
12R4	49037	43129	87.95	42618	41007	83.62	37176	75.81
12R5	5100	4435	86.96	4332	4267	83.67	4239	83.12
13R1	78187	68495	87.6	68211	67781	86.69	67272	86.04
13R3	196660	174047	88.5	173145	169770	86.33	123743	62.92
13R4	31527	26856	85.18	26654	26339	83.54	26253	83.27
13R5	89009	79274	89.06	79021	77855	87.47	65108	73.15
14R1	97706	85531	87.54	85006	83548	85.51	78720	80.57
14R2	78301	69450	88.7	68930	67006	85.57	59677	76.21
14R3	1059	760	71.77	721	703	66.38	703	66.38
14R4	70557	62375	88.4	61831	60343	85.52	55397	78.51
14R5	116	105	90.52	73	57	49.14	47	40.52
15R2	50619	44255	87.43	43754	42689	84.33	41745	82.47
15R4	191025	167242	87.55	166531	163825	85.76	150610	78.84
16R1	42038	36686	87.27	36289	35355	84.1	33201	78.98
16R2	119429	105377	88.23	104800	102440	85.77	87388	73.17
16R3	61802	54324	87.9	53863	52783	85.41	50630	81.92
16R4	75500	65916	87.31	65438	63839	84.55	58246	77.15
16R5	94265	83666	88.76	83375	82609	87.63	79180	84
17R1	50453	42407	84.05	42223	41862	82.97	41651	82.55
17R2	60410	52725	87.28	52400	51688	85.56	51332	84.97
17R3	75858	65995	87	65370	63221	83.34	57451	75.73
17R5	76433	67061	87.74	66541	65126	85.21	61027	79.84
18R1	73400	62661	85.37	62322	61756	84.14	60783	82.81
18R2	70899	63398	89.42	63112	62618	88.32	61455	86.68

18R3	79479	68590	86.3	68362	67860	85.38	67281	84.65
19R2	74150	64770	87.35	64690	64512	87	60076	81.02
19R3	30764	27044	87.91	26642	25675	83.46	23977	77.94
19R4	36195	31825	87.93	31657	31453	86.9	31329	86.56
19R5	18164	15945	87.78	15817	15675	86.3	15621	86
1R2	18627	16427	88.19	15957	14788	79.39	11949	64.15
1R3	50154	44317	88.36	43691	41737	83.22	31741	63.29
1R4	73271	64818	88.46	64186	62061	84.7	54731	74.7
1R5	97595	86349	88.48	85644	83424	85.48	74640	76.48
20R1	87092	76155	87.44	75701	74195	85.19	68872	79.08
20R2	72803	63040	86.59	62462	60579	83.21	56229	77.23
20R3	15207	13283	87.35	13135	12914	84.92	12696	83.49
20R5	107156	94950	88.61	94247	92192	86.04	73735	68.81
2R2	63308	55316	87.38	54856	53556	84.6	50439	79.67
2R3	64461	56831	88.16	56105	53918	83.64	45813	71.07
2R4	81520	71685	87.94	71106	69473	85.22	63744	78.19
2R5	46761	40719	87.08	40579	40338	86.26	40235	86.04
3R3	57417	49084	85.49	48791	48296	84.11	48112	83.79
3R4	72308	63499	87.82	62970	61311	84.79	56380	77.97
3R5	76008	67313	88.56	66614	64235	84.51	55284	72.73
4R1	68788	59910	87.09	59535	58634	85.24	56760	82.51
4R3	54228	47743	88.04	47371	46059	84.94	39619	73.06
4R4	23046	20298	88.08	20009	19577	84.95	19151	83.1
4R5	29312	25802	88.03	25516	24817	84.66	23876	81.45
5R2	44198	38988	88.21	38628	37632	85.14	34817	78.78
5R3	117491	103372	87.98	102665	100337	85.4	89798	76.43
5R5	62594	54411	86.93	53958	52480	83.84	48906	78.13
6R2	51814	45631	88.07	45386	44834	86.53	44062	85.04
6R3	59402	51610	86.88	51168	50209	84.52	48775	82.11
6R4	26873	23655	88.03	23449	23060	85.81	22574	84
6R5	111762	98300	87.95	97853	96427	86.28	93335	83.51
7R1	56125	49083	87.45	48891	48611	86.61	48393	86.22
7R2	73769	64416	87.32	64002	63207	85.68	61451	83.3
7R3	35098	30972	88.24	30635	29862	85.08	26283	74.88
7R4	31429	27712	88.17	27377	26416	84.05	24681	78.53
8R1	77411	67395	87.06	66838	64964	83.92	59648	77.05
9R1	50976	44191	86.69	43874	43174	84.69	41996	82.38
9R2	30780	26409	85.8	26146	25606	83.19	24897	80.89
9R3	74355	65801	88.5	65612	65039	87.47	59640	80.21
9R4	33822	29696	87.8	29461	29157	86.21	28824	85.22
9R5	58023	49996	86.17	49601	48730	83.98	47120	81.21

A

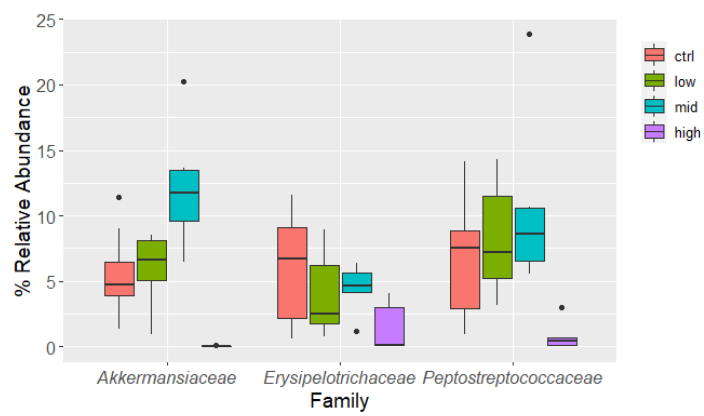


B

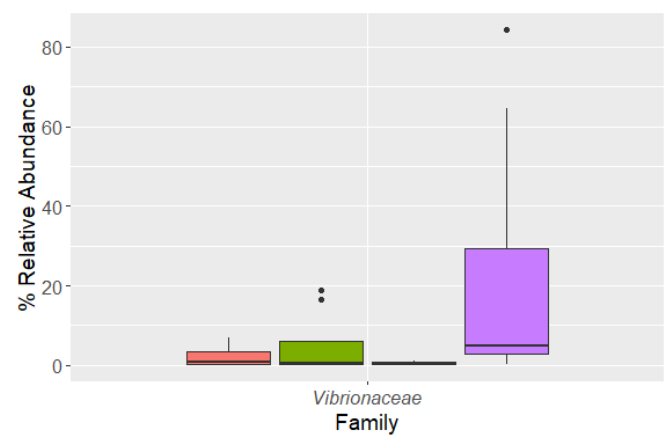


**Appendix G** | (A) Top 10 most abundant families in female fish that were significantly reduced in the high exposure group compared to controls. (B) Relative abundance of family level taxa that were significantly more abundant in the high exposure group compared to controls in females. (Chapter 3)

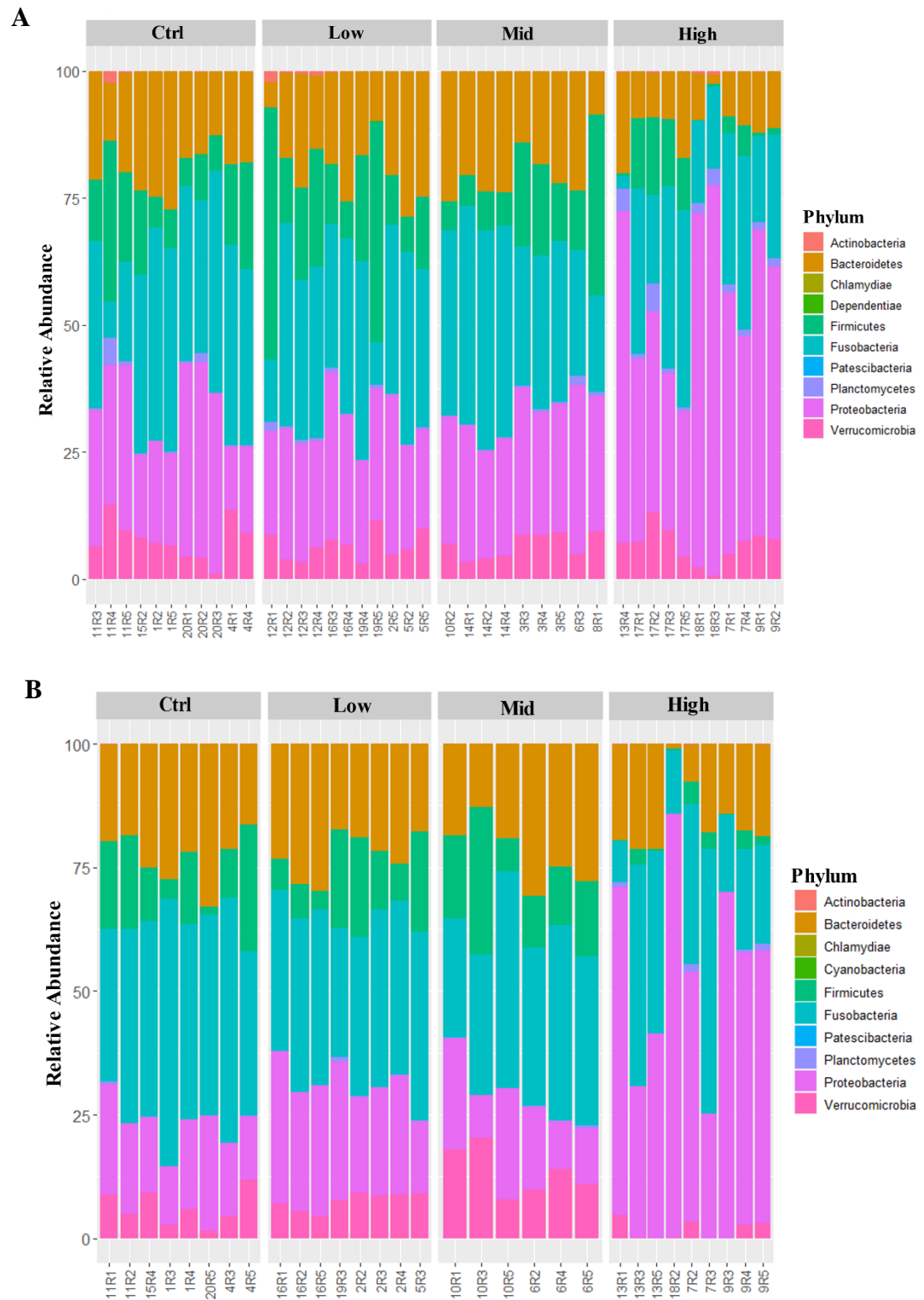
A



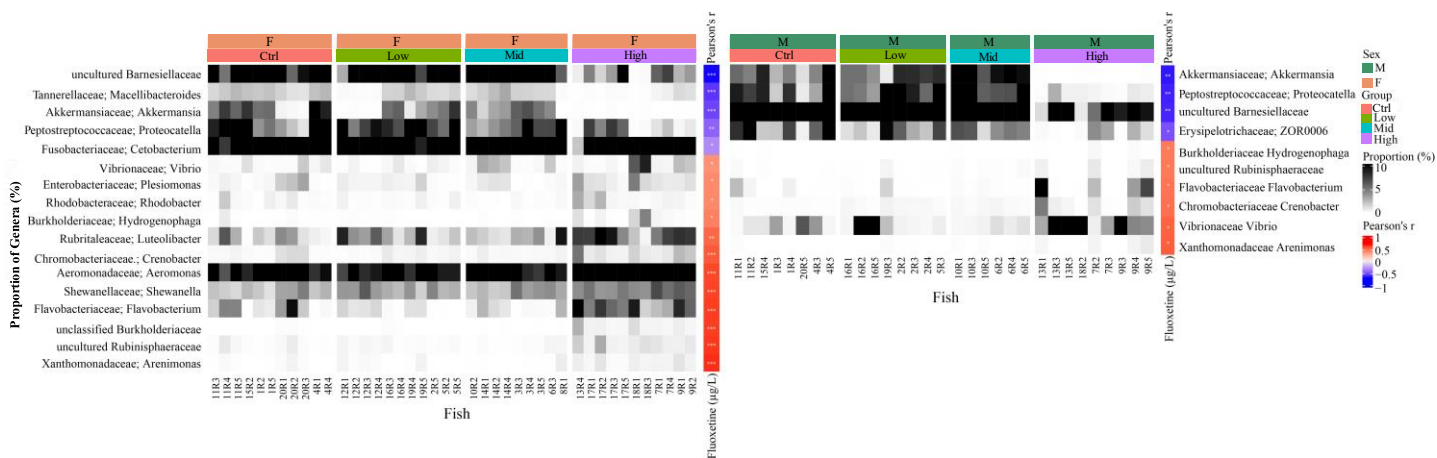
B



**Appendix H** | Relative abundance of family level taxa that are significantly reduced in the high exposure group compared to the control in males (A). Relative abundance of family level taxa that is significantly more abundant in the high exposure group compared to controls in males (Chapter 3).



**Appendix I** Relative abundances of top 10 Phylum level taxa in (A) females and (B) males.



**Appendix J|** Genera level heatmaps, of females (A) and males (B), representing the correlation between abundance of genera level taxa and fluoxetine concentrations. Greyscale indicates proportion of taxa, the darker the more abundant. Concentrations of fluoxetine include ctrl, low, mid and high. Pearson's correlation coefficient depicted in the blue-red scale, positive correlation (higher fluoxetine concentration and higher abundance of taxa) is shown in red while a negative correlation (high fluoxetine and lower abundance of ASVs) is shown in blue. (Chapter 3)





**Appendix L**| Summary of statistics from the Neighborhood Selection Network analyzer for male and female fish.

<b>Network analysis</b>		
<b>Summary of Statistics</b>	<b>Females</b>	<b>Males</b>
Number of nodes	209	171
Number of edges	562	339
Avg. number of neighbors	5,378	4,238
Network diameter	8	11
Network radius	5	6
Characteristic path length	3.52	3.949
Clustering coefficient	0.16	0.206
Network density	0.026	0.027
Network heterogeneity	0.673	0.792
Network centralization	0.187	0.221
Connected components	1	12
Analysis time (sec)	0.019	0.045

**Appendix M**| ASSESSING WHITE BLOOD CELL COUNTS AS AN INDICATOR FOR  
IMMUNOMODULATION BY FLUOXETINE

## 1. Introduction

The gut microbiome can influence the immune system of its host (Aidy et al., 2014; Chung et al., 2012; Dinan and Cryan, 2017; and Montalban-Arques et al., 2015). Gut microbiota are critical during the development and maturation of immune cells including stimulation of an immune response through antigen production (Chung et al., 2012) and molecular communication via cytokines and hormones (Aidy et al., 2014). Several immune cells can even produce neurotransmitters which can also influence gut bacteria, and the CNS, potentiating mood and behavioral responses (Aidy et al., 2014). Immune cells, including B, T, eosinophils, and basophils, express several types of serotonin transporters and any changes of these receptors can greatly influence the immune system (Coates et al., 2017). With evidence that the immune system produces and responds to neurotransmitters such as serotonin and the importance of the microbiome on immune responses, it is valuable to assess how fluoxetine may modulate the immune system and how immune system responses to fluoxetine may influence the gut microbiome, or vice-versa (fluoxetine modulation of the gut microbiome may influence the immune system).

Considering the importance of neurotransmitters in immune system responses, few studies have been conducted within the field of ecotoxicology to identify how SSRIs may affect immune function. However, there has been some speculation, including, how an increase of circulating serotonin due to fluoxetine exposure may cause beneficial changes to the immune system or potentiate autoimmune diseases (Brooks et al., 2003a). The few ecotoxicology studies concerning immune responses to fluoxetine found fluoxetine can change  $\text{Ca}^{2+}$  homeostasis, hamper cell viability, disrupt neurotransmitter signal transduction, and cause a hormesis effect on hemocyte counts in bivalves (Munari et al., 2014; Shi et al., 2019). Studies have been conducted looking at mammalian immune responses to fluoxetine. A review of these findings by Frick and Rapanelli (2013) identified SSRIs to increase natural killer cell activity, suppress bacterial antigen presentation of dendritic cells to T cells, modulate cytokine production, and reduce production of tumor necrosis factor- $\alpha$ . Fluoxetine was also found to increase T cell proliferation at low concentrations while causing an inhibitory effect at high doses, potentially through nonspecific toxicity (Frick and Rapanelli, 2013). With few studies conducted in the realm of ecotoxicology and the importance of serotonin on the immune system, it is essential to assess

how the immune function of fathead minnows may be affected by fluoxetine. The objective of this study was to run a white blood cell (WBC) count to identify any abnormalities in the number types of cells observed after fish were exposed to three different concentrations of fluoxetine. The null hypothesis for this study was that there would be no differences in the type of white blood cells identified or counted after fish were exposed to any concentration of fluoxetine.

## 2. Material and methods

### 2.1. General Methods

After a 28-day fluoxetine exposure to fathead minnows with aqueous concentrations of 0.01, 10 and 100  $\mu\text{g/L}$ , fish were euthanized, and blood was taken from the caudal vein to assess the immune system response to fluoxetine via white blood cell quantification. For in-depth methods regarding the fish exposure and fluoxetine quantification parameters see chapter 3.

### 2.2. Blood smears

After anesthetization with MS-222, the caudal fin was cut and a capillary tube was placed at the base of the caudal vein to allow for blood to enter the tube. A drop of blood was placed on a glass microscope slide. Using a second slide at a 45-degree angle, blood was smeared across the slide. The slide was let to dry and then dipped in methanol to fix the blood and placed in a slide box until further analysis. Before cell counting, slides were stained using the Hema 3™ Manual Staining System and Stat Pack following manufacturers guidelines (Fisher Scientific, Middleton, VA).

### 2.3. White blood cell counting

White blood cell counting took place using an Olympus BX41 compound microscope. Total leukocyte counts were done at 40x in the monolayer. Leukocytes were counted within ten fields per slide and the average was then determined. A Fudge Factor of 2,000 for the 40x objective was multiplied to the average white blood cell count to estimate the number of cells per  $\mu\text{L}$ . A 100-cell leukocyte differential was performed under oil immersion 100x lens within the monolayer. Running the slide in a zig-zag pattern all leukocytes were differentiated until 100 cells were counted. Methods were performed based on Rodak's Hematology: Clinical Principles and Applications (Rodak, 2016). Leukocytes were categorized by their shape and affinity to the dye and classified referencing Exotic Animal Hematology and Cytology (Campbell, 2015). Both

the total leukocyte count and the leukocyte differentials were conducted twice for better accuracy.

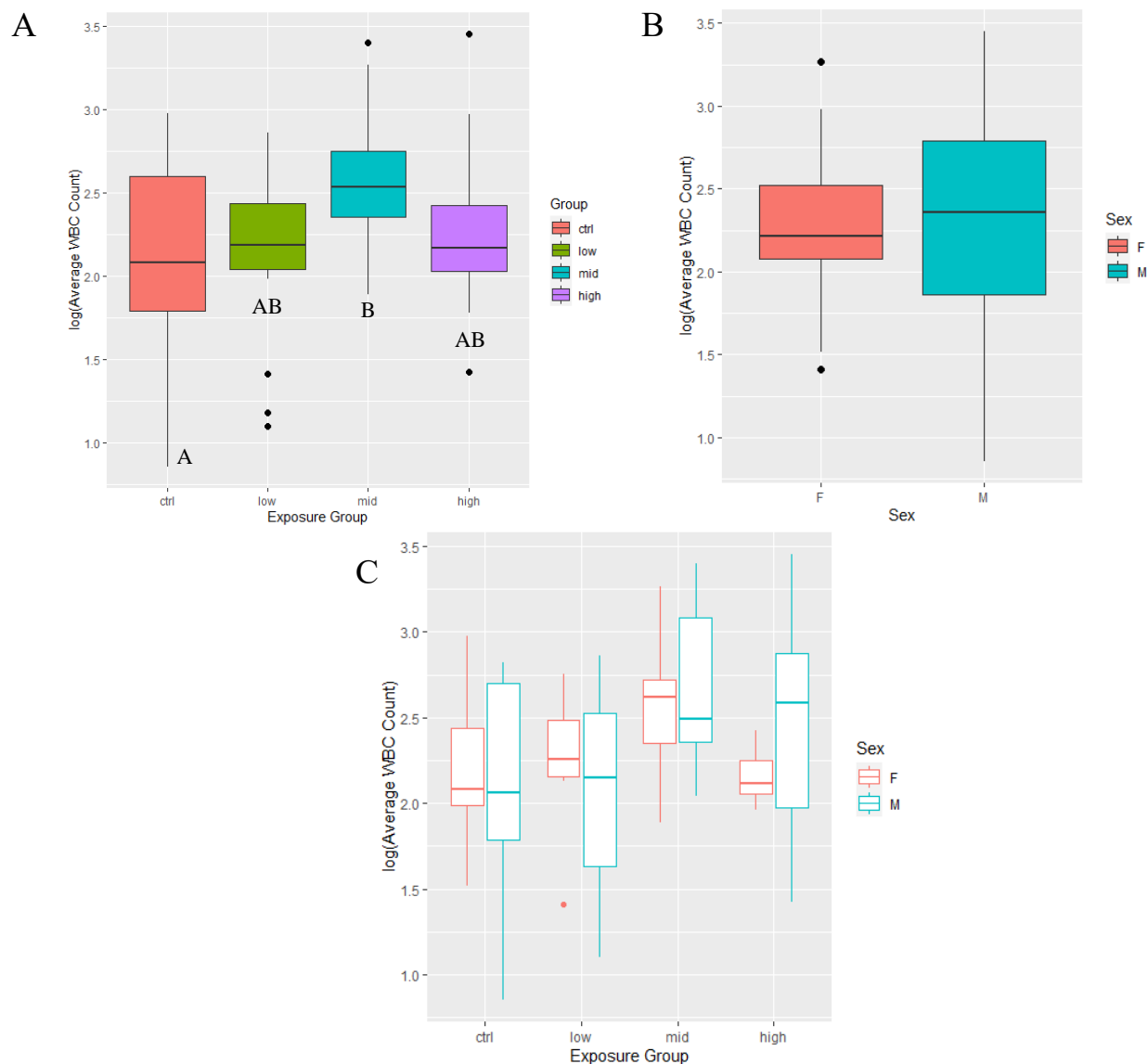
### 3. Statistics

Differences between total white blood cell counts and fluoxetine exposure concentrations were determined using an analysis of variance (ANOVA) with a Tukey HSD test (if the ANOVA held a  $p < 0.05$ ) to determine significance. By observing a Residual vs. Fitted plot and a quantile-quantile plot, as well as a Levene's test and Shapiro-wilk test, homogeneity of variance and normal distribution were determined before continuing on with the analysis. If these two assumptions were not met, tests were re-run on the logged transformed dataset. Because sex is known to have an effect on the immune system (Modrá et al., 1998; Rizzetto et al., 2018), a Welch's t-test was performed to identify if sex plays a role in total white blood cell counts. Again, if normal distribution and homogeneity of variance were not met, tests were conducted on the log-transformed data. All statistical analyses were run in R Statistical Language v 4.0.3 (R Core Team, 2020).

### 4. Results

Sex of fish did not play a role in total white blood cell count. Using the log-transformation of the data, a Welch's t-test revealed no significant difference between males and females when it came to total white blood cell counts (Figure 1, B).

A significant difference was found when comparing total white blood cell counts to fluoxetine exposure groups. The group exposed to the middle concentration (10 µg/L, nominal concentration) was found to have a significantly higher WBC count on average compared to fish in the control group ( $p = 0.03$ ). Although not statistically significant, the middle exposure group had a notably higher white blood cell count compared to the low group ( $p = 0.057$ ). No difference was found between the high and middle group (Figure 1A).



**Appendix M.1|** Assessment of log-transformed average white blood cell counts with fluoxetine exposure groups. The middle exposure group was found to have a higher white blood cell count than controls (A). Log transformed white blood cell counts in relation to sex of fish (B). Separation of sexes within each exposure group compared to log-transformed average white blood cell counts (C).

## 5. Discussion

A potential hormesis or biphasic response was identified when comparing total white blood cell counts to fluoxetine exposure; the middle exposure group was found to have a higher number of white blood cells on average compared to the control group while there was a slight

difference in number of WBCs comparing the middle and low exposure groups. No differences were found when comparing the high and middle groups. The results of this study, although predictive, are difficult to make any solid conclusions from. White blood cell counts can give an overall estimate of an immune system response and can help identify if there is an infection, inflammation or other non-specific immune responses within the body (George-Gay and Parker, 2003), however, it is much more insightful to run white blood cell differentials, where each type of WBC is counted (Blumenreich, 1990; Fazio, 2019; Wester et al., 1994). For example, an increase in neutrophils is linked to bacterial and fungal infections and inflammatory diseases (Campbell, 2015; Ghosh et al., 2016). Furthermore, identifying the ratio between lymphocytes and neutrophils is found to be a critical observation where toxic substances have been shown to decrease this ratio in fish species as reviewed by Modra et al., (1998). Unfortunately, due to difficulties in differentiating several types of immune cells in the blood of our study species and the lack of data on several fish species including fathead minnow immune cell averages and appearance (Fazio, 2019; Palić et al., 2005; Thomas et al., 1999), confidence was lacking in the data collected from the blood cell differentials, thus it was not used in the analysis.

Even with differential white blood cell counts there are still biases, importantly, counting error, when it comes to manual cell counting (Fazio, 2019; Garrey and Bryan, 1935). Other, more accurate, tools have been developed to better address immunotoxic responses. As reviewed by Rehberger et al., 2017, there are several different ways to predict immune system responses to toxicants including genomic assessments, where the identification of immune-related genes is conducted, namely cytokines, including interleukins and tumor necrosis factor. A few studies have even used transcriptomic approaches to assess immune system function and pathways (Shelley et al., 2012; Thornton Hampton et al., 2020). Other assessments used to address immune responses to toxicants include phagocytic activity identification using fluorescent-labeled beads and flow cytometry (Müller et al., 2009) and the use of nitro blue tetrazolium to identify respiratory bursts intended to kill pathogens (Kollner et al., 2002; Rehberger et al., 2017).

There is currently little information regarding the effects of fluoxetine on aquatic organisms' immune systems, however, there is an important need to investigate this further due to our understanding of the significance of serotonin on the immune system. Though

immunotoxicology is not without its challenges it is important to differentiate direct and indirect effects of toxicants on the immune system. Furthermore, the resting immune system may not show any significant perturbations but may indeed be impacted during a pathogen challenge (Rehberger et al., 2017; Thornton Hampton et al., 2020). Because of the complexity of the immune system, there is not just one test that is the most adequate to understand immunotoxicity, instead multiple tests may be conducted to estimate effects of the immune system (Rehberger et al., 2017). The study conducted here was merely a first insight into the implications fluoxetine may have on the immune system, but it is in no way conclusive and must be expounded upon using assays previously discussed to better identify if SSRIs hold modulatory abilities on the immune system.

Assessments of the immune system in combination with the gut microbiome in ecotoxicological studies can give us a better perspective on how the two may be interacting, for they both are critical in intestinal health and inflammatory responses (Yoo et al., 2020). Future work should be considered using a blended approach to better assess the impact of xenobiotics, such as SSRIs, on the microbiome in combination and relation to the immune system. This will give further insight into whether both systems may be perturbed and if there are direct or indirect effects associated with the xenobiotic of interest.



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